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(54) Title: METHOD FOR CONTROLLING SEED GERMINATION USING SOYBEAN ACYL COA OXIDASE SEQUENCES

(57) Abstract

A method is provided for the selective control of seed germination, wherein germination can be inhibited by expression of a germination inhibitor and, subsequently, germination can be induced via an inducible promoter that is operably linked to a germination restorer. Also disclosed are plants, plant cells, seeds and DNA constructs that are genetically engineered for control of seed germination.

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METHOD FOR CONTROLLING SEED GERMINATION USING SOYBEAN ACYL COA OXIDASE SEQUENCES

BACKGROUND OF THE INVENTION

This invention is directed to a method for controlling seed germination, as well as to DNA constructs, plants, plant cells and seeds genetically engineered for control of seed germination.

Biotechnological research has provided valuable tools for engineering novel and improved traits into a variety of commercial crops. This typically involves the introduction/transformation of foreign genetic material into plant cells followed by the regeneration of the plant cells into transgenic plants. The foreign genetic material generally comprises one or more recombinant DNA constructs which can promote or inhibit the expression of a protein of interest, depending upon the design of the transgene. In this way, it is possible to manipulate certain plant characteristics for the purpose of achieving agronomically desirable traits which were previously not attainable, or that were possible only through more expensive or laborious procedures.

One plant characteristic that may be addressed by a genetic-based approach is germination control. For example, it would be desirable to control crop outcrossing and volunteer seeds with selective germination control. The displacement of many plant species beyond their intended location of cultivation can occur as pollen is carried away, *e.g.*, by wind, birds or small mammals, thereby allowing pollination of the same or related species. In this way, crop species can outcross with related weed species such that the progeny seed are fertile. Plants from such seed can themselves assume the status of weeds, and may grow at times or in areas which are unintended and undesirable. The seeds may remain in subsequent crops as volunteers, *i.e.*, seeds which germinate in places or at times when they were not intended to germinate, for example, in non-field conditions or during post-harvest crop rotation.

An additional problem relates to the inappropriate timing of germination. Seed dormancy, *i.e.*, the inhibition of germination, is not fully functional in certain plant species,

particularly in cereals such as wheat and rice. Seed from such species can initiate the germination process prematurely while still on the plant in a process known as preharvest sprouting. This condition can be exacerbated by environmental conditions such as humidity and temperature. Preharvest sprouting has unfortunate consequences in that it can compromise crop quality and yield.

WO94/03619 discloses a method of controlling plant development by providing transgenic plants containing in their genomes two or more recombinant DNA constructs. A first DNA construct comprises an externally inducible promoter linked to a gene encoding a repressor protein. The second DNA construct contains a plant developmentally regulated promoter and an operator region linked to a disrupter protein gene (*e.g.*, a cytotoxin, recombinase, or antisense). In the presence of an inducer, the first DNA construct is expressed and the resulting repressor protein then negatively regulates the expression of the second DNA molecule by binding to the operator region. Thus, in this system, continual presence of inducer is required in order for plant development to proceed normally. If inducer is withdrawn, plant development is then disrupted. Such an approach, unfortunately, requires tightly regulated repressor-operator systems and developmentally specific promoters.

Alternative methods for the effective, selective control of seed germination would therefore be highly desired, particularly to prevent or minimize outcrossing, seed volunteers, and preharvest sprouting.

SUMMARY OF THE INVENTION

The invention is generally directed to a method of controlling seed germination that uses a plant, plant cell or seed having a genome comprising (a) a promoter operably linked to a first DNA sequence and a 3' untranslated region, wherein the first DNA sequence encodes a germination inhibitor; and (b) an inducible promoter operably linked to second DNA sequence and a 3' untranslated region, wherein the second DNA sequence encodes a

germination restorer. Seed germination may be selectively controlled by expressing the first DNA sequence to inhibit germination and, subsequently, by inducing the inducible promoter to allow expression of the second DNA sequence to restore seed germination.

In a preferred embodiment, the germination inhibitor comprises a protein for inhibiting germination. Alternatively, the germination inhibitor may comprise an antisense molecule, a co-suppression molecule containing sequences homologous to endogenous gene sequences, or a ribozyme, in each case capable of inhibiting the level or function of an endogenous mRNA or protein. The germination restorer preferably comprises an antisense RNA molecule capable of inhibiting the expression of the germination inhibitor or a protein that is functionally equivalent *in planta* to a protein necessary for germination which is inhibited by the germination inhibitor.

The invention is also directed to a method of controlling seed germination comprising (i) providing a seed having a genome comprising a promoter operably linked to a DNA sequence and a 3' non-translated region, wherein the DNA sequence encodes a germination inhibitor that inhibits the seed's production of a compound necessary for germination; and (ii) restoring seed germination by providing to the seed another compound that restores germination.

The invention is further directed to DNA constructs, plants. plant cells and seeds having DNA elements comprising (a) a promoter operably linked to a first DNA sequence and a 3' untranslated region, wherein the first DNA sequence encodes a germination inhibitor; and (b) an inducible promoter operably linked to second DNA sequence and a 3' untranslated region, wherein the second DNA sequence encodes a germination restorer.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the specification and are included to further demonstrate certain aspects of the invention. The invention may be better understood by

reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1 represents a vector map of plasmid pMON29400.

FIG. 2 represents a vector map of plasmid pMON29724.

FIG. 3 represents a vector map of plasmid pMON29711.

FIG. 4 represents a vector map of plasmid pMON29725.

FIG. 5 represents a vector map of plasmid pMON29444.

FIG. 6 represents a vector map of plasmid pMON10098.

FIG. 7 represents a vector map of plasmid pMON29705.

FIG. 8 represents a vector map of plasmid pMON29403.

FIG. 9 represents a vector map of plasmid pMON29404.

FIG. 10 represents a vector map of plasmid pMON17227.

FIG. 11 represents a vector map of plasmid pMON29405.

FIG. 12 represents a vector map of plasmid pMON999.

FIG. 13 represents a vector map of plasmid pMON29726.

FIG. 14 represents a vector map of plasmid pMON29415.

FIG. 15 represents a vector map of plasmid pMON29727.

FIG. 16 represents a vector map of plasmid pMON29729.

FIG. 17 represents a vector map of plasmid pMON29728.

FIG. 18 represents a vector map of plasmid pMON25289.

FIG. 19 represents a vector map of plasmid pMON25291.

FIG. 20 represents a vector map of plasmid pMON25292.

FIG. 21 represents a vector map of plasmid pMON25290.

FIG. 22 represents a vector map of plasmid pMON25294.

FIG. 23 represents a vector map of plasmid pMON25293.

FIG. 24 represents a vector map of plasmid pMON33501.

FIG. 25 represents a vector map of plasmid pMON33502.

FIG. 26 represents a vector map of plasmid pMON19648.

FIG. 27 represents a vector map of plasmid pMON29407.

FIG. 28 represents a vector map of plasmid pMON29412.

FIG. 29 represents a vector map of plasmid pMON29408.

FIG. 30 represents a vector map of plasmid pMON29409.

FIG. 31 represents a vector map of plasmid pMON29410.

FIG. 32 represents a vector map of plasmid pMON29411.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

For purposes of this invention, several terms are defined below.

"Germination" refers to the plant germination process which, for purposes of this invention, includes uptake of water by the seeds (imbibition), elongation by the embryonic axis (the radicle), and all related seedling growth events which result in the establishment of a vigorous seedling.

"Germination inhibitor" refers to an RNA or protein capable of inhibiting one or more germination events, observable as compromised growth, inferior vigor, reduced root growth, delayed emergence, non-uniform germination, reduced viability, and/or reduced germination rate. The germination inhibitor, for example, may impair the production or use of a component necessary for germination or may enhance production of a component that impairs germination.

"Germination restorer" refers to a chemical, RNA or protein capable of restoring the impaired plant germination events caused by the germination inhibitor.

"Inducible promoter" refers to a promoter which is responsive to an externally administered inducer comprising a chemical or other stimulus. In the absence of inducer, the promoter of the second DNA molecule of the present invention is not substantially active; it is either not expressed at all or is expressed at levels which are insufficient to cause significant restoration of the impaired germination events caused by the first DNA molecule.

The method of controlling seed germination according to the invention generally involves a seed having a genome comprising (a) a promoter operably linked to a first DNA sequence and a 3' untranslated region, wherein the first DNA sequence encodes a germination inhibitor; and (b) an inducible promoter operably linked to second DNA sequence and a 3' untranslated region, wherein the second DNA sequence encodes a germination restorer. Seed germination may be selectively controlled by expressing the first DNA sequence to inhibit germination and, subsequently, by inducing the inducible promoter to allow expression of the second DNA sequence to restore seed germination.

The invention is further directed to DNA constructs, plants, plant cells and seeds having DNA elements comprising (a) a promoter operably linked to a first DNA sequence and a 3' untranslated region, wherein the first DNA sequence encodes a germination inhibitor; and (b) an inducible promoter operably linked to second DNA sequence and a 3' untranslated region, wherein the second DNA sequence encodes a germination restorer.

DNA constructs according to the invention include transformation vectors which are capable of introducing foreign DNA, such as the first and second DNA sequences, into plants. The design of such DNA constructs and plant transformation methods using such constructs may employ a variety of conventional techniques. Plant transformation vectors generally comprise one or more coding sequences of interest under the transcriptional control of 5' and 3' regulatory sequences, including a promoter, and a selectable marker. Typical regulatory sequences include a transcription initiation start site, an RNA processing signal, a transcription termination site, and/or a polyadenylation signal. Plant promoters can be inducible or constitutive, environmentally- or developmentally-regulated, or cell- or tissue-specific. Often-used promoters include the CaMV 35S promoter (Odell et al., *Nature* 313:810 (1985)), the enhanced CaMV 35S promoter, the Figwort Mosaic Virus (FMV) promoter (Richins et al., *NAR* 20:8451 (1987)), the mannopine synthase (*mas*) promoter, the nopaline synthase (*nos*) promoter, and the octopine synthase (*ocs*) promoter. Useful inducible promoters may include heat-shock promoters (Ou-Lee et al., *Proc. Natl. Acad. Sci. USA* 83:6815 (1986)); a nitrate-inducible promoter derived from the spinach nitrite reductase gene (Back et al., *Plant Mol. Biol.* 17:9 (1991)); hormone-inducible

promoters (Yamaguchi-Shinozaki et al., *Plant Mol. Biol.* 15:905 (1990); Kares et al., *Plant Mol. Biol.* 15:905 (1990)), and light-inducible promoters associated with the small subunit of RuBP carboxylase and LHCP gene families (Kuhlemeier et al., *Plant Cell* 1:471 (1989); Feinbaum et al., *Mol. Gen. Genet.* 226:449 (1991); Weisshaar et al., *EMBO J.* 10:1777 (1991); Lam and Chua, *Science* 248:471 (1990); Castresana et al., *EMBO J.* 7:1929 (1988); Schulze-Lefert et al., *EMBO J.* 8:651 (1989)), and others known in the art. Promoter hybrids can also be constructed to enhance transcriptional activity (Hoffman, U.S. Patent No. 5,106,739), or to combine desired transcriptional activity, inducibility and tissue specificity.

Representative vectors typically comprise, operably linked in sequence in the 5' to 3' direction, a promoter sequence that directs the transcription of a downstream heterologous structural DNA in a plant; optionally, a non-translated leader sequence; a nucleotide sequence that encodes a protein of interest; and a 3' non-translated region that encodes a termination/polyadenylation signal which functions in plant cells to cause the termination of transcription and the addition of polyadenylate nucleotides to the 3' end of the mRNA encoding said protein.

The promoters used in the DNA constructs (*i.e.*, chimeric/recombinant plant genes) of the invention may be modified, if desired, to affect their control characteristics. Promoters can be derived by means of ligation with operator regions, random or controlled mutagenesis, etc. Furthermore, the promoters may be altered to contain multiple "enhancer sequences" to assist in elevating gene expression. Examples of such enhancer sequences have been reported by Kay et al. (1987).

The RNA produced by a DNA construct of the present invention also contains a 5' non-translated leader sequence. This sequence can be derived from the promoter selected to express the gene, and can be specifically modified so as to increase translation of the mRNA. The 5' non-translated regions can also be obtained from viral RNA's, from suitable eukaryotic genes, or from a synthetic gene sequence. The present invention is not limited to constructs wherein the non-translated region is derived from the 5' non-translated sequence that accompanies the promoter sequence.

An antisense expression construct generally contains a DNA sequence which provides for the production of an RNA sequence which is complementary to a mRNA sequence sought to be inhibited. An antisense construct can be generated in a number of ways, provided it is capable of being transcribed into RNA which is complementary to and capable of blocking the translation of mRNA produced by the endogenous gene. Typically, the construct is generated by inverting some or all of the coding region of the endogenous gene to allow for transcription of its complement. The antisense RNA molecule should contain sufficient complementarity in sequence and sufficient length of sequence to the endogenous mRNA molecule in order to achieve the desired inhibition/inactivation. Various types of antisense constructs can be used to carry out the specific embodiments of the present invention. Thus, the endogenous mRNA targeted for inhibition/inactivation will determine the promoter required to carry out the invention.

A cosuppression construct generally contains a DNA sequence which is capable of inhibiting the accumulation of normal levels of mRNA or protein (Meyer, P., *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 47:23-48 (1989)). The DNA sequence contains homology to endogenous genes. The construct could consist of homology to endogenous promoters and influence their transcription. Alternatively, the cosuppression construct can consist of a sequence containing the full length or partial mRNA sequence in 5' to 3' sense orientation and is usually joined to promoter and 3' terminator sequence that allow for transcription into RNA.

Another alternative for decreasing expression of an endogenous gene is a ribozyme (Gerlach, W.L. et al., "Use of Plant Virus Satellite RNA Sequences to Control Gene Expression," *Viral Genes and Plant Pathogenesis* (T.P. Pirone and J.G. Shaw, eds., Springer Verlag, NY) pp. 177-186 (1990)). The ribozyme consists of sequences homologous to the mRNA to be inhibited and the sequences required for catalytic activity of the ribozyme. This specific ribozyme RNA is joined to promoter and 3' terminator sequences that allow for transcription into RNA.

A variety of different methods can be employed to introduce such vectors into plant protoplasts, cells, callus tissue, leaf discs, meristems, etc., to generate transgenic plants, including *Agrobacterium*-mediated transformation, particle gun delivery, microinjection, electroporation, polyethylene glycol-mediated protoplast transformation, liposome-mediated transformation, etc. (reviewed in Potrykus, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 42:205-225 (1991)). In general, transgenic plants comprising cells containing the DNA

molecules of the present invention can be produced by transforming plant cells with a DNA construct as described above via any of the foregoing methods; selecting plant cells that have been transformed on a selective medium; regenerating plant cells that have been transformed to produce differentiated plants; and selecting a transformed plant which expresses the recombinant DNA molecule(s) and exhibits the desired germination inhibition/inactivation.

The encoding DNAs can be introduced either in a single transformation event (all necessary DNAs present on the same vector), a co-transformation event (all necessary DNAs present on separate vectors that are introduced into plants or plant cells simultaneously), by independent transformation events (all necessary DNAs present on separate vectors that are introduced into plants or plant cells independently) or by re-transformation (transforming an already transformed line generated by a single transformation, co-transformation, or independent transformation events). Traditional breeding methods, when applicable, can subsequently be used to incorporate the entire pathway into a single plant.

Specific methods for transforming a wide variety of dicots and obtaining transgenic plants are well documented in the literature (see Gasser and Fraley (1989); Fisk and Dandekar (1993); Christou (1994), and the references cited therein), including methods for cotton (U.S. Patent No. 5,004,863; U.S. Patent No. 5,159,135; U.S. Patent No. 5,518,908), soybean (U.S. Patent No. 5,569,834; U.S. Patent No. 5,416,011; McCabe et al. (1988)), Brassica (U.S. Patent No.5,463,174), *Arabidopsis* (Bechtold et al. (1993)), peanut (Cheng et al.(1996); Livingstone et al. (1995); Mckently et al. (1995)), and peas (Grant et al. (1995); Schroeder et al. (1993); De Kathen et al. (1990)).

Successful transformation and plant regeneration have been achieved in numerous monocots including asparagus (*Asparagus officinalis*; Bytebier et al. (1987); barley (*Hordeum vulgarae*; Wan and Lemaux (1994)); maize (*Zea mays*; Armstrong, C.L. et al., *Crop Science* 35:550-557 (1995); Rhodes et al. (1988); Gordon-Kamm et al. (1990); Fromm et al. (1990); Koziel et al. (1993)); oats (*Avena sativa*; Somers et al. (1992)); orchardgrass

(*Dactylis glomerata*; Horn et al. (1988)); rice (*Oryza sativa*, including indica and japonica varieties; Toriyama et al. (1988); Zhang et al. (1988A); Luo and Wu (1988); Zhang and Wu (1988B); Christou et al. (1991)); rye (*Secale cereale*; De la Pena et al. (1987)); sorghum (*Sorghum bicolor*; Cassas et al. (1993)); sugar cane (*Saccharum* spp.; Bower and Birch (1992)); tall fescue (*Festuca arundinacea*; Wang et al. (1992)); turfgrass (*Agrostis palustris*; Zhong et al. (1993)); and wheat (*Triticum aestivum*; Vasil et al. (1992); Weeks et al. (1993); and Becker et al. (1994)).

The methods and DNA constructs according to the invention are applicable to any plant, plant cell or seed for which it may be desirable to control germination. The invention is particularly applicable for use in barley, canola, corn, cotton, oat, pea, peanut, rice, sorghum, soybean, sugarcane, and wheat.

The reversible germination system of the present invention comprises two general components: 1) a way to disrupt or inhibit normal germination in situations where germination is not desirable and 2) a way to recover or rescue normal germination when germination is desired. The first component could be accomplished by inhibiting genes or functions that are essential for germination or early seedling growth, vigor, or yield. When normal germination is required, the second component in the form of a seed coating or spray application would be used which would consist of compound(s) capable of replacing the missing gene or its products directly or inducing another gene to complement the missing gene or its products. Another way to obtain germination control is to enhance expression or overexpress a gene product which itself inhibits germination or early seedling growth/vigor/yield. The gene product could keep a seed in a dormant state or could influence vital processes in the germination/early seedling development phase. In this case, the rescue treatment would function to inhibit the inhibitor gene or its product/function or induce a secondary pathway or process that would relieve the block or bypass it.

In one embodiment, the invention generally involves four genetic components for purposes of selective seed germination control: 1) a gene which acts as an inhibitor of germination (germination inhibitor), 2) a specific promoter to regulate the germination

inhibition gene, for example a germination enhanced promoter, 3) a rescue gene (germination restorer), and 4) a regulatable promoter (inducible promoter). A further component of the present invention is the rescue treatment used to restore germination, preferably a chemical seed treatment, a foliar application, or a nonchemical induction such as heat, cold, light, etc.

Therefore, in accordance with one embodiment of the present invention there is provided a method of controlling seed germination in transgenic plants wherein a first recombinant DNA molecule functions to cause a germination inhibition phenotype. There are several biochemical pathways or processes that, if interrupted, would have an adverse affect on germination or seedling vigor. Additionally, there are genes that when overexpressed would also have an inhibitory effect on germination. In this invention, a preferred mechanism to inhibit germination is to interfere with the ability of the seed to utilize its lipid storage reserves. A number of enzymes are involved in this process which function to break down the lipid and/ or reconvert the intermediates to carbohydrate in the process of gluconeogenesis. These enzymes and processes could be inhibited by interfering with the gene, its expression, or activity. Mutation breeding could be used to identify plants missing a given gene or function. Antisense, co-suppression, or ribozymes could be used to interfere with normal accumulation of mRNA encoding germination enzymes. Alternatively, dominant negative or other proteinaceous inhibitors could be overexpressed to decrease a germination enzyme's function. Additionally, enzymes which divert important pathway intermediates could be used to decrease the flux through the lipid utilization pathway and thereby inhibit germination.

A preferred enzyme to be inhibited by the first DNA molecule of the present invention is acyl CoA Oxidase (ACOX). ACOX is the first enzyme of peroxisomal ß-oxidation of fatty acids which catalyzes the oxidation of acyl CoAs to 2-trans-enoyl-CoAs. It donates electrons directly to molecular oxygen, thereby producing H_2O_2. ACOX genes have been isolated from rat (Miyazawa et al. (1987)), humans (Aoyama et al. (1994)), *Candida tropicalis* (Okazaki et al. (1986)), *Candida maltosa* (Hill et al. (1988)), *Saccharomyces cerevisiae*, (Dmochowska et al. (1990)) and barley (Grossi et al. (1995)). In

addition, two *Arabidopsis* ESTs have been identified which show significant homology to ACOXs from other organisms (Newman et al. (1994); Clone ID# 35H7T7 and 5F12T7P).

Similarly other enzymes in the lipid mobilization pathway could be inhibited by the first DNA molecule of the present invention in order to block germination, for example: 1) lipases which cause hydrolysis of the fatty acid ester bonds in the triacylglycerols stored in the seed, ultimately resulting in the formation of glycerol and free fatty acids. Lipase activity is absent in ungerminated seeds and increases rapidly in postgermination (Huang (1993)); 2) other enzymes in the fatty acid β-oxidation pathway such as a) acyl coA synthetase which activates free fatty acids to acyl CoAs, b) multifunctional protein, a single protein which catalyzes the hydration of 2-trans-enoyl-CoA to 3-hydroxyacyl-CoA and its subsequent oxidation to 3-oxoacyl-coA, and c) thiolase which cleaves 3-oxoacyl CoA to acyl CoA and acetyl CoA by a thiolytic cleavage and appears to be the rate-limiting step in the ß-oxidation pathway; and 3) enzymes in the glyoxylate cycle, which is involved in gluconeogenesis such as isocitrate lyase and malate synthase which are coordinately expressed in canola cotyledons and axes from 1 to 6 days after seed imbibition (Comai et al. (1989)). A similar pattern of expression is seen in cotton, cucumber and castor.

The specificity of the first DNA molecule of the present invention can be provided by inhibiting a germination specific or intensive process or gene or by interfering with a nonspecific process in a germination specific way. For example, if a function to be knocked out is germination specific, it could be knocked out using constitutive viral promoters such as the cauliflower mosaic virus 35S or figwort mosaic virus 35S promoters or cellular promoters such as a ubiquitin, actin, or cyclophilin promoter. If the function is essential for germination, but its gene expression is not limited to this time, a specific promoter could be used. The preferred promoter would be only expressed in the appropriate tissues and cells at the appropriate developmental time to inhibit the germination enzyme only during germination or early seedling growth. Germination-enhanced promoters have been isolated from genes encoding the glyoxysomal enzymes isocitrate lyase and malate synthase from several plant species (Zhang et al. (1994); Reynolds and Smith (1995); Comai et al. (1992)). Other promoters include SIP-seedling imbibition protein (Heck, G.R., "Regulation of Gene

Expression During Barley Seed Germination," PhD thesis, Washington University, St. Louis, Missouri (1991); Heck, G.R. et al., "Gibberellin-repressible gene expression in the barley aleurone layer," *Plant Molecular Biology* 30:611-623 (1996)) and others such as a cysteine endopeptidase promoter (Yamauchi et al. (1996)). Additionally, promoters could be isolated from other genes whose mRNAs appear to accumulate specifically during the germination process, for example class I ß-1,3-glucanase B from tobacco (Vogeli-Lange, et al. (1994)), canola cDNAs CA25, CA8, AX92 (Harada et al. (1988); Dietrich et al. (1992)), lipid transfer protein (Sossountzove et al. (1991)), or rice serine carboxypeptidases (Washio and Ishikawa (1994)).

In a further embodiment of the present invention, there is provided a means to rescue the germination inhibition phenotype by use of a chemical or other inducer which can circumvent the effects of the first DNA molecule. A compound which directly complements the blocked enzyme could be utilized. In the case of blockage of the lipid utilization process, plants with loss of ACOX or other lipid utilization enzyme activities would be expected to be restored to normal growth by supplementation with sucrose, the pathway's end product, or with other intermediates/products of the pathway such as citrate, malate, succinate or glyoxylate. Alternatively, other carbon sources could be utilized as a rescue agent to provide energy for the growing seedling normally provided by the products of lipid degradation. Such compounds can be used to restore germination since they complement the germination inhibition phenotype caused by the first DNA molecule.

In a further embodiment of the present invention, there is provided a chemical or other inductive treatment which can induce the expression a complementing gene. Thus, a second DNA molecule of the present invention comprises an inducible promoter operably linked to a DNA sequence which functions to restore germination. The gene could complement the blocked step by directly replacing it or by circumventing the need for that enzyme or part of the pathway. Alternatively, the inducer could drive expression of an inhibitor which would repress the germination inhibition construct or inhibit the activity of its product(s).

For example, where an antisense sequence against a plant acyl coA oxidase gene is used to inhibit seed germination, an ACOX gene, preferably from a non-plant source (*e.g.*, fungal or mammalian), could be used in the second DNA molecule in order to rescue the germination inhibition. For a nonplant gene to replace the antisensed plant ACOX gene's activity, it must be able to be expressed at the right levels and in the right subcellular location. In addition, it must replace the required enzymatic activity with the functionally relevant substrate specificity. ACOX genes have been isolated from a number of nonplant sources such as rat (Miyazawa et al. (1987)), human (Varanasi et al. (1994)), and yeast (Okazaki et al. (1986)). An inducible gene expression system regulating the nonplant ACOX expression, for example a chemically induced promoter, would provide for expression at the appropriate time.

Alternatively, if a specific portion of a gene is used in the antisense, for example a 5' untranslated leader or 3' terminator, a rescue gene that lacked the portion in the antisense construct could be overexpressed. This would allow for a plant ACOX to be used. For example, if the 3' untranslated region of a plant ACOX mRNA were used to effectively inhibit seed germination, the rescue could be achieved using the same or similar plant ACOX expressed as a fusion gene containing the plant ACOX with a nonACOX 3' terminator such as the nopaline synthase terminator (Fraley et al. (1983)) or pea E9 rbc-S 3' end (Coruzzi et al. (1984)). A potential advantage would be a closer match to the endogenous substrate specificity and possibly better plant expression than a heterologous ACOX coding sequence. A related approach would utilize a full or partial synthetic gene which would encode a functional ACOX with the appropriate substrate specificity to replace that knocked out by the inhibitory (*e.g.*, antisense) construct but with a lack of sufficient nucleotide homology to be downregulated itself by the antisense sequence.

Yet another alternative is to use a second DNA molecule which functions to repress the antigermination effects of the first DNA molecule by decreasing its expression by antisense (or cosuppression, ribozyme, etc.). If the first DNA molecule comprises an antisense construct, a unique region of the mRNA expressed would act as a target of the

second DNA molecule. The second DNA molecule is preferably regulated by a chemically inducible promoter or other regulated gene expression system.

Plant genes are induced by a variety of signals, some of which may be mimicked by addition of a chemical stimulus or by other environmental stimulus. Safener-induced promoters could be used, for example the 27Kd subunit of glutathione-S-transferase II (WO90/08826); other chemically induced promoters such as the soybean GH2/4 promoter (Ulmasov et al. (1995)) which is induced by a variety of chemicals, or hormonally induced promoters such as α-amylase or HVA22 (Shen and Ho (1995)). In addition, prokaryotic transcriptional regulatory systems can be utilized in plants. For example, the tetracycline repressor system from transposon Tn10 can be utilized as a repressible (Gatz and Quail (1988); Gatz et al. (1991)) or inducible system (Weinmann et al. (1994)). Alternatively, the *E.coli* lac operator/repressor system (Wilde et al. (1992)) could be utilized. In addition, eukaryotic regulated expression systems could also be utilized such as the glucocorticoid inducible system (Aoyama and Chua (1997)), copper inducible system (Vadim and Reynolds), nitrate inducible promoter (Back et al. (1991)).

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLES

Example 1. Isolation and characterization of the soybean ACOX gene family and its expression

a. Isolation of ACOX cDNA clones. Amino acid sequences for the rat (Miyazawa, et al. (1987)), human (Varanasi et al. (1994)), and yeast (Okazaki et al. (1986)) Acyl CoA Oxidase (ACOX) genes were aligned to identify regions of homology. In addition, partial sequences from *Arabidopsis* (obtained from the Expressed Sequence Tag database and identified based on homology to the sequences indicated above) and barley (Grossi et al. (1995)) were aligned. The regions with highest conservation were identified and degenerate oligonucleotides were designed to encode these amino acid sequences. The primers contained the following nucleotide sequences where R=A+G, Y=C+T, K=G+T, S=G+C, W=A+T, and N=A+G+C+T :

Hom2a:	5'- TIT TYC CIY TIY TIG CIW SNG C -3' (SEQ ID NO.1)
Hom4:	5'- GIA ARY TIT GYG GIG GIC AYG G -3' (SEQ ID NO.2)
Hom8:	5'- RTA ACR TTI CCR TCR TAI CKN C -3' (SEQ ID NO.3)

Reverse transcription-polymerase chain reaction (RT-PCR) was used to obtain soybean ACOX sequences. RNA was isolated from soybean cotyledons from 4 day old seedlings and first strand cDNA was made in a reaction containing using the Superscript pre-amplification kit (Gibco-BRL) using conditions recommended by the manufacturer. The products of the reverse transcription reaction were then amplified using PCR amplification conditions. After an initial 3 minute 94°C denaturation step, 30 cycles were run, each with 94°C, 20 second denaturation, followed by 1 minute annealing, followed by 2 minutes extension at 72°C. The annealing temperature of the first two cycles was 58°C and after every other cycle, the annealing temperature was lowered 1°C to a final temperature 44°C. Ten additional cycles were run with a 94°C, 20 sec; 43°C, 1 min; and 72°C, 2 min temperature regime.

The PCR reactions yielded a 0.7-0.8Kb band and were purified by agarose gel electrophoresis. The putative ACOX fragments were cloned into the TA vector (Invitrogen)

to form pMON29400 (FIG.1), pMON29401, and pMON29402. Nucleotide sequence was obtained from each insert (SEQ ID NOS.4-6). Nucleotide sequence homology alignments were performed (Gish and David (1993); Altschul et al. (1990)) which indicated that the soybean fragments were significantly similar to the ACOX sequences from other organisms.

To obtain a full-length clone, the soybean ACOX PCR fragment from pMON29400 was used to screen a λgt10 library prepared from 10 day old soybean seedlings. An initial screen of 300,000 plaques resulted in the isolation of 5 partial overlapping clones. A second screen of 800,000 plaques was carried out with the longest clone from the first screen resulting in the isolation of 10 clones. Sequence analysis of all 15 clones revealed that the clones fell into two distinct classes. Of the 15 clones, 11 belonged to one class (Class I) and 4 to the second class (Class II).

The longest clone (clone #20.1a) obtained was 2.1 kb long and belonged to Class I. The cDNA insert from this clone was amplified with λgt10-lft and λgt10-rt primers (Clontech Laboratories, Palo Alto, CA) which flank the EcoRI cloning site in λgt10 and the resulting PCR fragment was cloned into the TA vector (Invitrogen) to form pMON29724 (FIG.2). This ACOX clone was found to be incomplete at the 3' end and therefore it was joined to another Class I clone containing the complete 3' end. The 3' end containing clone (clone #1a) was also amplified as described above and cloned into the TA vector (Invitrogen) to form pMON29711 (FIG.3). To join the two clones, pMON29724 was digested with NotI/XbaI and pMON29711 was digested with XbaI/KpnI. The resulting two fragments were then inserted between the NotI/KpnI sites of pMON29711 in a triple ligation. The resulting construct pMON29725 (FIG.4) contained a full-length soybean ACOX Class I clone containing 156 bp of the 5' UTR, the entire coding region and 130 bp of the 3' UTR. The sequence obtained from this clone is shown in SEQ ID NO.7. The longest clone belonging to Class II (Clone#2a) was a partial clone and its sequence is shown in SEQ ID NO.8. This Class II clone shows 87% identity with the full-length Class I clone.

The following table indicates the sequence homology between the full-length soybean ACOX (soy ACOXI) and other known ACOX sequences:

Table 1: Comparison between soy ACOXI and other ACOXs

	Nucleotide level	Amino acid level
Soy ACOXII	87% (1019 nt)	88% (340 aa)
Arabidopsis EST ID #35H7T7	76% (977 nt)	82% (326aa)
Barley	67% (393 nt)	56% (157 aa)
C. tropicalis POX5	55% (200 nt)	39% (358 aa)
C. tropicalis POX4	51% (783 nt)	34% (557 aa)
Rat	59% (1057 nt)	47% (556 aa)
Arabidopsis EST ID #SF12T7P	52% (620 nt)	30% (346 aa)

b. Characterization of ACOX Gene Family in Soybean: Southern analysis was performed in order to understand the ACOX gene family in soybean. Soybean genomic DNA was digested with five different restriction enzymes - EcoRI, BamHI, HindIII, SpeI and SspI. The digested DNA samples were electrophoresed on a 0.8% agarose gel. The gel was first treated with 0.25M HCl for 15 minutes. After rinsing with dH_2O, the gel was treated with denaturation buffer containing 1.5M NaCl and 0.5M NaOH for 30 minutes. The gel was rinsed several times with dH_20 and then soaked in neutralization buffer containing 1.5 M NaCl, 0.5M Tris HCl pH 7 and 0.001M EDTA for 15 minutes. The gel was blotted in 20X SSC (3M NaCl, 0.3M Sodium Citrate) onto Hybond N nylon membrane (Amersham). The DNA was UV crosslinked to the membrane using the UV Stratalinker (Stratagene). The Southern blot was probed with a partial ACOX cDNA fragment which shows high homology to both classes of soybean ACOXs. The probe was prepared by random priming using the Rediprime DNA labelling system from Amersham. Hybridization was performed at 37°C for 20 hours in 50% formamide, 6X SSC, 5X Denhardts solution, 0.2% SDS and 100 µg/ml denatured salmon sperm DNA. The blot was washed by first rinsing in 2X SSC, 0.5% SDS at RT, followed by two 15 minute washes at RT in 2X SSC, 0.1% SDS, followed by two 30 minute washes at 52°C in 1X SSC, 0.1% SDS. The blot was exposed with intensifying screen at -70°C to Kodak X-OMAT film. The blot was re-washed two times in a high stringency buffer consisting of 0.1X SSC, 0.1% SDS at 65°C for 30 minutes each. Two to three bands of hybridization were detected for each digest under high stringency conditions. This suggests that at least two to three ACOX

genes exist in soybean. Low stringency conditions revealed the presence of one to three extra bands in each digest, suggesting the existence of more distantly related ACOX genes.

The soybean ACOX Class I and II show a high degree of nucleotide sequence homology, 76 and 77% respectively, to an *Arabidopsis* ACOX EST (EST ID#35H7T7P). However, a second *Arabidopsis* ACOX EST (EST ID#5F12T7P) exists which is only 60% homologous EST ID#35H7T7P. The soybean ACOXI and ACOXII sequences have 52% and 54% identity, respectively, to EST ID#5F12T7P. In order to determine if ACOX EST ID#5F12T7P-like sequences exist in soybean, Southern analysis was performed using this EST as the probe. All experimental conditions were the same as described above except changes were made in the hybridization and wash conditions. Hybridization was performed at 37°C for 24 hours in 35% formamide, 5X SSC, 5X Denhardts solution, 0.1% SDS and 100 µg/ml denatured salmon sperm DNA. The blot was washed two times at RT for 15 minutes each in 2X SSC, 0.2% SDS; followed by two 20 minute washes at 37°C in 2X SSC, 0.2% SDS; followed by one final wash for 30 minutes at 37[C1]°C in 0.2X SSC, 0.2% SDS. One to two bands of hybridization were obtained in each lane, of which at least one is unique relative to the above Southern. Therefore, a third class of soybean ACOX (soy ACOXIII) could exist in soybean. These data indicate that the plant ACOX genes are highly related and that the expression of all three classes could be down regulated in a similar way.

*c. **ACOX expression during soybean seedling growth.*** Northern analysis was performed to determine the temporal and spatial expression pattern of ACOX during soybean seedling growth. Total RNA was isolated from soybean seedlings starting at 6 hours after imbibition until 7 days after imbibition. For the 6 hour, 12 hour and 24 hour samples, the seedlings were dissected into cotyledons and axis. The 2 day and 3 day samples were divided into cotyledons, axis and roots. The 5 day and 7 day samples were divided into cotyledons, epicotyls, hypocotyls and roots. RNA was isolated according to the procedure of Rochester et al. (1986). After denaturation in 50% formamide and 2.18M formaldehyde, the RNA samples were separated on a 1% agarose gel. The gel was transferred in 10X SSC onto Hybond N nylon membrane (Amersham) which was then UV

crosslinked using the UV Stratalinker (Stratagene). The Northern blot was probed with a partial ACOX class I cDNA which shows homology to two classes of soybean ACOXs. The probe was prepared by random priming using the RTS Radprime DNA labeling system from GIBCO BRL. Hybridization was performed for 20 hours at 42°C in 50% formamide, 6X SSC, 5X Denhardts solution, 0.2% SDS and 100 µg/ml denatured salmon sperm DNA. The blot was washed by first rinsing at room temperature in 2X SSC, 0.5% SDS; followed by two 15 minute washes at room temperature in 2X SSC, 0.1% SDS; followed by two 45 minute washes at 55°C in 0.1X SSC, 0.1% SDS. The blot was exposed with an intensifying screen at -70°C to Kodak X-OMAT film.

No ACOX expression was seen in dry seeds. ACOX mRNA was detected as early as 6 hours after imbibition. In the early seedling samples (until 2 days after imbibition), the mRNA level was higher in the growing axis than in the cotyledons. In the later seedling samples (until 7 days after imbibition), ACOX mRNA was detected in all tissues examined (cotyledons, hypocotyls, epicotyls and roots). Maximum expression was seen in hypocotyl tissue from 5 day old seedlings. Expression levels decreased after 5 days. In addition, the ACOX mRNA was found to accumulate in mature leaves and also during late seed development. In all cases, the mRNA was approximately 2.8 kb in size. The results are summarized in the following Table:

Table 2: ACOX mRNA levels during soybean seedling growth

Stage	Whole tissue	Cotyledon	Axis	Epicotyl	Hypocotyl	Root
Developing seed - early	+					
Developing seed - late	+					
Mature seed		-	-			
6 HAI		+	+++			
12 HAI		+	+			
1 DAI		+	++			
2 DAI		+	+++			
3 DAI		+	++++			++++
5 DAI		+		++	++++	++
7 DAI		+		+++	++	+
Leaf	+++					

Note: HAI = Hours after imbibition
DAI = Days after imbibition

*d. **Antibody against soybean ACOX protein.*** Polyclonal antibodies were produced using an *E. coli*- produced antigen essentially as described in Ausubel et al. (1994). A full-length class I ACOX coding sequence was constructed essentially as described in Example 1a and inserted into pET30a(+) (Novagen) under the T7/lac promoter to form pMON29444 (FIG.5).

pMON29444 was then transformed into the *E. coli* strain, BL21(DE3) (Novagen). The soybean ACOX-I protein was then overexpressed by adding IPTG to 1mM in the growth medium, LB broth, and purified by using Novagen's His·Tag Ni^{2+} chelation resin (Cat. #69670). The purified ACOX-I protein was injected into rabbit for antibody production (Scientific Association Inc., St. Louis).

The antisera was used to detect ACOX expression pattern during soybean seedling growth. Total protein was extracted (extraction buffer: 150 mM KPO4, pH 7.5; 10% glycerol; 1 mM EDTA; 5 mM β-mecaptoethanol; 0.1% triton; 2 mM Pefabloc) from

soybean dry seeds, cotyledons (one, two, three, five and seven day after imbibition(DAI)), axis (one, two and three DAI), roots (three, five and seven DAI), and epicotyls and hypocotyls (five and seven DAI). 20 μg of each total protein sample was separated on a 10-20% polyacrylamide gradient gel (BioRAD) and transferred onto ECL nitrocellulose membrane (Amersham). A 1:30,000 dilution of primary antisera was used to detect ACOX protein using the ECL detection system (Amersham).

A 75Kd protein was detected in all the samples in this assay and the protein size was consistent with the size deduced from the amino acid sequence of ACOX protein. The ACOX protein was at low levels in dry seeds and the level increased after two DAI. ACOX protein level was higher in axis, root, epicotyls and hypocotyls and lower in cotyledons. The maximal accumulation was detected in epicotyls and hypocotyls of five and seven day old seedling. ACOX protein was also found in mature leaf tissues, but the levels were lower than that in five and seven day old epicotyls and hypocotyls. The results are summarized in the following table:

Table 3: ACOX protein levels during soybean seedling growth

Stage	Whole tissue	Cotyledon	Axis	Epicotyl	Hypocotyl	Root
Mature seed	+					
1 DAI		+	++			
2 DAI		+	+++			
3 DAI		+	++++			+++
5 DAI		+++		++++	+++++	+++
7 DAI		+		+++++	+++++	++
Leaf	+++					

Note: DAI = Days after imbibition

Example 2. Construction of antisense ACOX plant transformation vectors and production of transgenic *Arabidopsis* plants

a. Construction of antisense ACOX plant transformation vectors. An *Arabidopsis* EST (Expressed Sequence tag) which showed significant homology to known ACOX sequences was obtained from the *Arabidopsis* Biological Resource Center at Ohio State (EST ID# 35H7T7) (Newman et al. (1994)). In order to construct an antisense vector, the EST DNA was digested with BamHI and EcoRI and the resulting 1 kb fragment was inserted into pMON10098 (FIG.6) between the BglII/EcoRI sites to create pMON29705 (FIG.7). pMON29705 contained the enhanced 35S promoter, antisense-*Arabidopsis* ACOX, and an E9 terminator. pMON29705 also contained a kanamycin cassette for constitutive expression in plants for use in kanamycin selection and two border sequences for T-DNA transfer. pMON29705 was introduced into *Agrobacterium tumefaciens* and utilized in *Arabidopsis* transformation by vacuum infiltration (Bechtold et al. (1993)).

b. Germination analysis of transgenic plants. The V1 seeds obtained from 7 independent plants which had been vacuum infiltrated were pooled, sterilized by bleach fumes overnight under vacuum and plated on MS medium (Murashige and Skoog basal medium containing Gamborg's vitamins, Sigma # M-0404) containing 50 ug/ml kanamycin to select for plants containing and expressing the selectable marker. The kanamycin resistant seedlings were transferred to soil, grown to maturity and the resulting V2 seeds were collected. The V2 seeds from 100 independent lines were analyzed for germination phenotype. Fifty seeds from each line were sterilized as above and plated on MS medium to determine the effect of antisense ACOX expression on seedling growth. 24 of the 100 lines analyzed showed abnormal seedling growth, particularly observable as a significant reduction in root length and vigor. Thus, the ACOX antisense vector has a significant effect on germination and early seedling growth.

Since sucrose is the end product of the lipid utilization pathway, plants with loss of ACOX or other lipid utilization enzyme activities would be expected to be restored to normal growth by supplementation with sucrose, the pathway's end product. To demonstrate the reversiblity of the ACOX-inhibition phenotype in these lines, the phenotype

of these lines was determined on sucrose containing media. Fifty seeds from each line were treated as above and plated on MS medium containing 20 mM sucrose. All 24 lines showed improved growth on sucrose containing media; most lines were totally restored to wild type growth.

Thus, the sucrose-dependent early seedling growth phenotype suggests that ACOX is required for normal early seedling growth in oil seed plants such as *Arabidopsis*, soybean, cotton and canola.

*c. **Analysis of antisense expression**.* Northern analysis was performed on the 24 germination impaired lines in order to determine the levels of expression of the antisense transcript and to determine if there was any decrease in ACOX mRNA levels. The seeds from these lines were sterilized as above and plated on MS medium containing 20 mM sucrose and 50 ug/ml kanamycin. After 10 days, the kanamycin resistant seedlings were collected and approximately 100 mg of tissue was used to extract total RNA. Total RNA was extracted with the RNeasy Plant Total RNA kit by Qiagen (Catalog # 74904) as per the manufacturer's instructions. Fifteen micrograms of total RNA was separated on a formaldehyde gel and transferred to Hybond N nylon membrane (Amersham). Wild type RNA from seedlings grown in similar conditions was included as control. The blot was probed with *Arabidopsis* ACOX EST (ID # 35H7T7) using the same hybridization and wash conditions as described in Example 1c.

Of the 24 lines analyzed, 21 lines showed a 1.5 kb transcript corresponding to the antisense transcript in addition to the 2.8 kb endogenous ACOX transcript. Of these 20, 13 showed very strong expression of the antisense mRNA, approximately 50-100 fold in excess of the endogenous transcript. Of the 13 antisense expressing lines, 6 lines showed lower levels of the endogenous ACOX mRNA compared to the wild type control. The data for these 6 lines is summarized in Table 4. This data confirms that expression of antisense ACOX mRNA results in a significant reduction in the endogenous ACOX mRNA levels, thereby affecting a germination inhibition phenotype.

Table 4: Antisense xpression in anti-ACOX *Arabidopsis* transgenic plants

Line#	Percent Germination		Antisense expression	Endogenous ACOX mRNA levels
	-Sucrose	+Sucrose		
Wild type	83	100	-	100%
29705 #6	54	88	+	20%
29705 #7	45	90	+	20%
29705 #47	54	100	+	15%
29705 #52	45	93	+	10%
29705 #58	39	96	+	10%
29705 #96	46	100	+	10%

Example 3. Construction of antisense ACOX plant transformation vectors and production of transgenic soybean plants

a. Construction of antisense ACOX plant transformation vectors. Antisense vectors for germination enhanced expression in plants were constructed using one of the soybean ACOX fragments. pMON29400 (FIG.1) was digested using EcoRI and the ends of the 0.8Kb soybean ACOX fragment made blunt by filling in the 5' EcoRI overhangs with Klenow polymerase followed by purification by gel electrophoresis. The fragment was then inserted into pMON29403 (FIG.8) into the StuI site between the *Brassica* isocitrate lyase (ICL) promoter (Zhang et al. (1993); Zhang et al. (1994); Zhang et al. (1996)) and the nopaline synthase 3' terminator (NOS, Fraley et al. (1983); Depicker et al. (1982)) to form pMON29404 (FIG.9). The entire plant expression cassette from pMON29404, containing ICL promoter, anti-ACOX, and NOS 3' end, was excised on a single NotI fragment and inserted into the NotI site in pMON17227 (FIG.10) to form pMON29405 (FIG.11). In addition to the P-ICL/antiACOX/NOS sequences, pMON29405 also contains a CP4 cassette for constitutive expression in plants for use in glyphosate selection and two border sequences for T-DNA transfer into the plant chromosome. PMON29405 is introduced into *Agrobacterium tumefaciens* and utilized in soybean transformations as described (U.S. Patent Nos. 5,416,011 and 5,569,834).

Antisense vectors were constructed which contained the full-length soybean ACOXI sequence in antisense orientation under the control of a constitutive promoter (35S) and a

germination enhanced promoter (Isocitrate lyase). DNA from pMON29725 was amplified using the following primers:

29725-Kpn: CGG GGT ACC GGT GAA TCC AGA G (SEQ ID NO.9)
29725-Bam: CAA AGG ATC CTT CCA TGC CTC (SEQ ID NO.10)
29725-Stu: CAG TTC CGG ATA CAC CTA ATT G (SEQ ID NO.11)

PCR reaction was performed using 1 ng of pMON29725 DNA and 29725-Kpn and 29725-Bam as 5' and 3' primers respectively. The PCR conditions consisted of one denaturation cycle at 92°C for 2 minutes followed by 30 cycles consisting of 92°C denaturation for 1 minute, 55°C annealing for 1 minute 30 seconds, 68°C extension for 2 minutes. The resulting 2 kb fragment was purified by electrophoresis, digested with KpnI/BamHI and inserted between the KpnI/BglII sites of pMON999 (FIG.12) to create pMON29726 (FIG.13). The fragment was thus placed between the enhanced 35S promoter and the nopaline synthase 3' terminator (NOS). This fragment contained the 5' untranslated region and the coding region of soy ACOXI. However, it lacked 84 of the coding region at the 3' end and also the 3' untranslated region.

PCR reaction was also performed using 1 ng of pMON29725 DNA and 29725-Kpn and 29725-Stu as 5' and 3' primers respectively. The PCR conditions used were the same as described above. The resulting 2.2 kb fragment was purified by electrophoresis, digested with KpnI/StuI and inserted between the KpnI/StuI sites of pMON29415 (FIG.14) to create pMON29727 (FIG.15). The fragment was thus placed between the Isocitrate lyase promoter and the NOS terminator. This fragment contained the 5' and 3' untranslated regions and the entire coding region of soy ACOXI.

The entire expression cassette in pMON29726 and pMON29727 was excised as a NotI fragment and inserted into the NotI site in pMON17227 (which contained a cassette for constitutive expression of CP4 EPSPS to allow for glyphosate selection in plant transformation) to form pMON29729 and pMON29728 respectively (FIGS.16 & 17). The two constructs, pMON29729 and pMON29278 were then introduced into *Agrobacterium* for plant transformation.

Glyphosate is employed as a selectable marker (Hinchee et al. (1994)) to identify transformed soybean tissue. Leaves of glyphosate-resistant soybean transformants (designated R_0 generation) are screened for CP4 EPSPS expression by ELISA (Padgette et al. (1995)). Seeds from R_0 CP4-positive plants (designated R1 seeds) are collected and germination, early seedling growth, lipid content, and other growth characteristics evaluated. In addition, levels of ACOX mRNA, protein, and enzyme activity are measured to determine the extent to which the antisense construct has altered the expression level of the endogenous ACOX gene.

Example 4. Expression of yeast ACOX in *Arabidopsis*.

a. Construction of yeast ACOX overexpression vectors. Sequences of the *Candida tropicalis* ACOX genes (POX4, POX5) were obtained using polymerase chain reaction based on published sequences (Okazaki et al. (1986)). *Candida tropicalis* was obtained from the American Type Culture Collection (ATCC) and was grown overnight in YPD media at 30°C. The cells were pelleted and DNA was isolated using the procedure described in Ausubel et al.(1994). PCR was performed using Extend Long Template PCR System Kit (Boehringer Mannheim) with 100ng yeast DNA template and the following primers:

CTR11: 5'- CAG ATC TTC ACG ACA TAA TG -3' (POX4) (SEQ ID NO.12)
CTR12: 5'- CGA GCT CTT CTA TTC TTA CTT GG -3' (POX4) (SEQ ID NO.13)
CTR21: 5'- CAG ATC TCG CTA TCA TGC CTA CGG -3' (POX5) (SEQ ID NO.14)
CTR22: 5'- CCT AGA GCT CTA TTA ACT GGA C -3' (POX5) (SEQ ID NO.15)

The PCR reaction conditions were:

One cycle of: 92°C, 2 min;
Ten cycles of: 92°C, 10 sec; 55°C, 30 sec; 68°C, 5 min;
Ten cycles of: 92°C, 10 sec; 55°C, 30 sec; 68°C, 5 min; ext 20sec

The 2.2Kb PCR fragment for POX4 and a 2.0 Kb fragment for POX5 were purified by 1% agarose gel electrophoresis and inserted into TA vector (Invitrogen) to form

pMON25289 (POX4; FIG.18) and pMON25291 (POX5; FIG.19). Nucleotide sequence was obtained to confirm that the PCR fragments were identical to the published sequences.

Vectors for overexpression of the yeast ACOX sequences in plants were constructed using the PCR fragments. The POX4 sequence was excised from pMON25289 as a SmaI-SacI fragment and ligated into the StuI-SacI sites in pMON999 to form pMON25292 (FIG.20). The POX5 sequence was excised from pMON25291 as a BglII-EcoRI fragment and ligated into the BglII-EcoRI sites in pMON19648 to form pMON25290 (FIG.21). Each of these vectors contained the CaMV 35S promoter with duplicated enhancer sequence (E35S) and nopaline synthase 3' terminator (NOS, Fraley et al. 1983; Depicker, et al. 1982) to drive overexpression of yeast ACOX genes in plants. The entire expression cassette in pMON25292 and pMON25290 was excised as a NotI fragment and inserted into the NotI site in pMON17227 (which contained a cassette for constitutive expression of the CP4 EPSPS to allow for glyphosate selection in plant transformation) to form pMON25294 (POX4; FIG.22) and pMON25293 (POX5; FIG.23). pMON25294 and pMON25293 were then introduced into *Agrobacterium* for plant transformation. Transgenic *Arabidopsis* plants were generated by the method of Bechtold et al.(1993).

b. Analysis of transgenic Arabidopsis. Polyclonal antibodies were produced against yeast ACOX proteins in rabbits immunized with *E. coli*-produced antigens. Full-length coding sequences of yeast ACOX genes, POX4 and POX5 were generated essentially as described before (Example 4(a)) and inserted into pET30a(+) vector (Novagen) under the T7/lac promoter to form pMON33501 (FIG.24) and pMON33502 (FIG.25).

The two constructs were then transformed into the *E. coli* strain, BL21(DE3) (Novagen). The yeast ACOX proteins were then overexpressed by adding IPTG to 1mM in the LB broth growth medium and purified by using Novagen's His·Tag Ni^{2+} chelation resin (Cat. #69670). The purified POX4 and POX5 proteins were injected into rabbits for antibody production (Scientific Association Inc., St. Louis).

The antisera against POX5 protein was used to detect POX5 overexpression in transgenic *Arabidopsis* plants transformed with pMON25293 (pEnhCaMV35S/POX5/NOS-3', Example 4a). Total protein was extracted from 100 mg of tissue with extraction buffer containing 50 mM Tris-HCl pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue and 10% glycerol. The protein samples were separated on a 10-20% polyacrylamide gradient gel (BioRAD) and transferred onto ECL nitrocellulose membrane (Amersham). A 1:1000 dilution of primary antisera was used to detect POX5 protein using the ECL detection system (Amersham).

A 75Kd protein was detected in all the samples from 14 *Arabidopsis* V2 lines analyzed and the protein size was consistent with the size of purified POX5 protein observed by others (Shimizu et al. (1979)). Transgenic *Arabidopsis* plants overexpressing the yeast POX5 protein showed no abnormal phentoypes during early seedling growth and throughout the development of the plants. This data demonstrates successful overproduction of a yeast ACOX in plants without any adverse effects on growth.

Transgenic soybean plants are made as described in Example 3. R0 plants as well as R1 seeds and seedlings are carefully monitered to determine if any adverse phenotypes are observed. R1 seed composition is analyzed to determine if there is any alteration in lipid, protein, or carbohydrate content. In addition, germination and early seedling growth characteristics are determined. POX 4 and POX5 expression is characterized by Northern and Western blot analyses. ACOX enzyme assays are performed with short, medium, and long chain fatty acid substrates (Shimizu et al. (1979)) to determine if the total ACOX enzyme activity has changed or if the profile of substrate specificity is altered.

Example 5. Construction of vectors for inducible expression of yeast ACOX genes

The GmHSP26(GH2/4) promoter region was identified from soybean by Ulmasov et al (1995) and a promoter-GUS fusion construct was shown to be induced by a wide variety of chemical agents in a tissue-specific and concentration-dependent manner in transgenic tobacco plants. PCR was used to isolate the GmHSP26(GH2/4) promoter sequences

utilizing primers HSP1 and HSP5 which contain sequences homologous to the promoter with restriction sites on the ends for cloning.

HSP1 GGT ACC AAG CTT AGG TTA CGA TCT CAA AAT CG (SEQ ID NO.16)
HSP5 CCA CCA TGG AGA TCT GCT ACA ATA CAA ACA ATG (SEQ ID NO.17)

Soybean genomic DNA was isolated using standard methods (Fedoroff, *Cell* 35:225-233 (1983)). 100 ng genomic soybean DNA was used as template in a reaction using the Expend Long Template PCR System Kit (Boehringer Mannheim).

The PCR reaction conditions were:

One cycle of: 92°C, 2 min;
Ten cycles of: 92°C, 10 sec; 55°C, 30 sec; 68°C, 5 min;
Ten cycles of: 92°C, 10 sec; 55°C, 30 sec; 68°C, 5 min; ext 20sec.

The 880 PCR fragment containing the HSP26 promoter was purified by 1% agarose gel electrophoresis, digested with HindIII and NcoI and ligated into the HindIII and NcoI sites of pMON19648 (FIG.26) to yield pMON29407 (Fig.27; P-HSP26/GUS/NOS 3'). Nucleotide sequence analysis of the PCR generated insert in pMON29407 was used to confirm that the sequence is intact. The entire expression cassette in pMON29407 was excised as a NotI fragment and inserted into the NotI site in pMON17227 (which contains a cassette for constitutive expression of the CP4 EPSPS to allow for glyphosate selection in plant transformation) to form pMON29412 (FIG.28).

Vectors for inducible expression of the yeast ACOX sequences were constructed using the PCR fragments described above. The HSP26 promoter sequence was excised from pMON29407 as an HindIII /BglIII fragment and ligated into the HindIII/BglII sites in pMON25292 and pMON25290 to replace the enhanced 35S promoter and to form pMON29408 (FIG.29; P-HSP26/POX4/NOS) and pMON29409 (FIG. 30; P-HSP26/POX5/NOS). The entire expression cassettes in pMON29408 and pMON29409 were excised as NotI fragments and each inserted into the NotI site in pMON17227 (which contains a cassette for constitutive expression of the CP4 EPSPS to allow for glyphosate selection in plant transformation) to form pMON29410 (FIG.31) and pMON29411

(FIG.32). pMON29410, pMON29411, and pMON29412 were introduced into *Agrobacterium tumefaciens* and utilized in plant transformations.

Glyphosate is employed as a selectable marker (Hinchee et al. (1994)) to identify transformed plant tissue. Leaves of glyphosate-resistant transformants (designated R_0 generation) are screened for CP4 EPSPS expression by ELISA (Padgette et al. (1995)). Seeds from R_0 CP4-positive plants (designated R1) are collected and germination, early seedling growth, lipid content, and other growth characteristics evaluated.

The expression pattern of the HSP26 promoter throughout the lifecycle of the plant is characterized in the presence and absence of a several inducers including: 2,4D (a synthetic auxin), heat, and 3-(dichloroacetyl)-5-(2-furanyl)-2,2-dimethyl-oxazolidine (furilazole). Detailed analysis of tissue and cell type distribution and timing of expression is obtained from germinating seedlings containing pMON29412 (P-HSP26/GUS).

The best inducer of the P-HSP26/GUS expression is utilized to characterize the lines containing an inducible ACOX construct such as pMON29410 and pMON29411. Leaves of Ro plants are treated with inducer and expression of ACOX utilizing short, medium, and long chain fatty acid substrates is assayed as described (Shimizu et al. (1979)). Several lines with good induction profiles are chosen for further analysis. These lines are allowed to self and also outcrossed to lines with reduced ACOX levels during germination due to the ACOX antisense construct pMON29405 (Example 3).

Example 6. Cross to create antisense ACOX::HSP26-POX4 and antisense ACOX::HSP26-POX5 lines

Progeny from the cross between pMON29405-containing (Example 3) and pMON29410- or pMON29411-containing lines (Example 5) are planted and the lines containing both constructs identified. Lines containing one of the constructs are retained as controls. The plants are allowed to produce seed for evaluation and to create plants homozygous for both transgenes. Seeds are germinated in the presence or absence of inducer. In the absence of inducer, germination inhibition phenotype is observed. In the

presence of inducer, seed treatment of fuvilazole, expression of POX4 or POX5 is turned on. The expression of yeast ACOX enyzme restores the ability of the seedling to break lipids and allows them to germinate and grow normally. In this way, the two constructs plus an inducer combine to form a chemically controllable seed germination system.

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(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

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(B) FILING DATE:
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(2) INFORMATION FOR SEQ ID NO:1:

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(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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(B) LOCATION: 2..17

(D) OTHER INFORMATION: /mod_base= i

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(B) TYPE: nucleic acid
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
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ACACTGATCA CTACCTTGGT TCAATTCTTG GACGTTACGA TGGAAATGTG TATCCGAAGA 2040

TGAACGAGGA GGCATGGAAG GATCCTTTGA ATGATTCAGT TGTTCCTGAT GGCTTTAAAG 2100

AGTATATTCA ACCGATGCTT AAGCAGCAAC TACGTAATGC TAGGCTGTAG TTAATTTTGT 2160

GGCAATGATG CTTTTGGCAT CTAAGAAATT TACCCAGACT ATTCTGATTT ACAACTCTTA 2220

ATAAAGTTGT GTTTGCCAGC TAGTAATTAC CATCGCAATT AGGTGTATCT GGAACTGGA 2279

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1147 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GAATTCCGGG GAATGAAATT TGGAAATGGA GCATATAACT CCATGGATAA TGGGATGCTA 60

AGGTTTGACC ATGTACGGAT TCCAAGAAAT CAAATGTTAA TGAGGGTTTC ACAGGTAACA 120

AGGGAAGGAA AATATGTACA ATCCAGTGTT CCACGACAAT TAGTCTATGG TACTATGGTA 180

TATGTAAGAC AAACAATTGT ATCTGATGCG TCAGTTGCTT TGTCGCGAGC AGTTTGCATT 240

GCTACAAGAT ATAGTGCTGT TCGAAGACAG TTTGGGTCAA AAGAGGGAGG TCTTGAGACA 300

CAGGTGATTG ATTATAAAAC GCAGCAAGCT AGGCTCTTCC CTTTGTTAGC TTCTGCCTAT 360

GCCTTCAGAT TTGTTGGTGA GTGGTTGAAA TGGCTTTATA TGGATGTGAT GAAAAGATTG 420

CAAGCCAGTG ATTTTTCAAC CTTACCTGAG GCTCATGCGT GCACTGCAGG GTTGAAGTCC 480

TTGACTACTT CAGCAACTGC TGATGGAATT GAGGAATGCC GCAAACTATG TGGTGGCCAT 540

GGTTACCTTT GCAGCAGTGG TCTCCCTGAG TTATTTGCAG TCTACATTCC TACCTGCACA 600

TATGAAGGAG ACAACACTGT GCTGCTTTTA CAGGTGGCTA GGCATCTCAT CAAGACTATT 660

TCTCAGTTGG GCTCCAGAAA CAAGCCTGTT GGTACAACAT CTTACATTGG ACGAGTGGAA 720

CAGCTTATGC AATATCGTTC TGATGTTCAG AAAGTGGAGG ATTGGCTGAA GCCTAATGCA 780

GTGTTGGGAG CATTTGAAGC TAGGGCTGCT AAGAAGGTGG TTGCTTGTGC TCAAAATCTC 840

AGCAAGTTTA CCAATCCCGA AGAAGGTTTC CAAGAACTCT CAGTCGATCT AGTTGAGGCA 900

GCTGTTGCTC ATTGCCAGTT AATTGTTGTT TCCAAATTTA TTGAGAAGTT GCAGCAAGAT 960

ATCCCTGGAA AGGGAGTGAA ACAGCAATTA GAACTTCTTT GTAGCATTTA CGCTTTGTTT 1020

CTTCTTCACT TTACTCATAT TCCGGAAATA CAGAAACAAA GAAATTCCAC GATCAAACTA 1080

CCAAATCCAA ATGAAATAGA CTAACAAACA ATAAGAAACC AAAATGCTTA ACTTTCCTCC 1140

GGAATTC 1147

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CGGGGTACCG GTGAATCCAG AG 22

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CAAAGGATCC TTCCATGCCT C 21

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CAGTTCCGGA TACACCTAAT TG 22

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CAGATCTTCA CGACATAATG 20

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CGAGCTCTTC TATTCTTACT TGG 23

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CAGATCTCGC TATCATGCCT ACGG 24

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CCTAGAGCTC TATTAACTGG AC 22

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GGTACCAAGC TTAGGTTACG ATCTCAAAAT CG 32

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CCACCATGGA GATCTGCTAC AATACAAACA ATG 33

H: 193649(45F501!.DOC)

WHAT IS CLAIMED IS:

1. A method of controlling seed germination, comprising:

(i) providing a seed having a genome comprising

(a) a promoter operably linked to a first DNA sequence and a 3' untranslated region, wherein the first DNA sequence encodes a germination inhibitor sequence; and

(b) an inducible promoter operably linked to a second DNA sequence and a 3' untranslated region, wherein the second DNA sequence is a germination restorer;

(ii) inducing the inducible promoter to restore seed germination.

2. The method of claim 1, wherein the germination inhibitor comprises a protein for inhibiting germination.

3. The method of claim 1, wherein the germination inhibitor is an antisense molecule, a co-suppression molecule containing sequences homologous to endogenous gene sequences, or a ribozyme, in each case capable of inhibiting the level or function of an endogenous mRNA or protein.

4. The method of claim 3, wherein the endogenous mRNA encodes a protein necessary for plant lipid mobilization.

5. The method of claim 4, wherein the endogenous mRNA encodes an acyl CoA oxidase enzyme.

6. The method of claim 5, wherein the endogenous mRNA encodes a soybean acyl CoA oxidase I (SEQ ID NO.7) or acyl CoA oxidase II (SEQ ID NO.8) mRNA.

7. The method of claim 1, wherein the promoter is a CaMV 35S, FMV 35S, ubiquitin, actin, cyclophilin, isocitrate lyase, malate synthase, SIP-seedling inhibition protein, cysteine

endopeptidase, tobacco class I ß-1,3-glucanase B, canola CA25, canola CA8, canola AX92, lipid transfer protein or rice serine carboxypeptidase promoter.

8. The method of claim 1, wherein the germination restorer comprises a protein that allows the seed to germinate.

9. The method of claim 8, wherein said protein is a yeast POX4 or yeast POX5 enzyme.

10. The method of claim 1, wherein the germination restorer comprises an antisense RNA molecule capable of inhibiting the expression of the germination inhibitor.

11. The method of claim 1, wherein the inducible promoter is derived from the 27Kd subunit of glutathione-S-transferase II, GH2/4, amylase, HVA22, tetracycline A promoter/operator, *E. coli* lac promoter/operator, glucocorticoid inducible promoter element, copper inducible promoter element or nitrate inducible promoter element.

12. The method of claim 3, wherein the germination restorer comprises a protein that is functionally equivalent *in planta* to the protein necessary for germination which is inhibited by the germination inhibitor.

13. A method of controlling seed germination, comprising:

(i) providing a seed having a genome comprising a promoter operably linked to a DNA sequence and a 3' non-translated region, wherein the DNA sequence encodes a germination inhibitor that inhibits the seed's production of a compound necessary for germination; and

(ii) restoring seed germination by providing to the seed another compound that restores germination.

14. The method of claim 13, wherein the germination inhibitor encodes a protein for inhibiting plant germination.

15. The method of claim 13, wherein the germination inhibitor is an antisense molecule, a co-suppression molecule containing sequences homologous to endogenous gene sequences, or a ribozyme in each case capable of inhibiting the level or function of an endogenous mRNA or protein.

16. The method of claim 15, wherein the endogenous mRNA encodes a protein necessary for plant lipid mobilization.

17. The method of claim 16, wherein the endogenous mRNA encodes an acyl CoA oxidase enzyme.

18. The method of claim 17, wherein the endogenous mRNA encodes a soybean acyl CoA oxidase I (SEQ ID NO.7) or acyl CoA oxidase II (SEQ ID NO.8) enzyme.

19. The method of claim 13, wherein the promoter is a CaMV 35S, FMV 35S, ubiquitin, actin, cyclophilin, isocitrate lyase, malate synthase, SIP-seedling inhibition protein, cysteine endopeptidase, tobacco class I ß-1,3-glucanase B, canola CA25, canola CA8, canola AX92, lipid transfer protein or rice serine carboxypeptidase promoter.

20. The method of claim 16, wherein the restorer compound is sucrose, malate, succinate, citrate or glyoxylate.

21. A plant, plant cell or seed having a genome comprising:

(i) a promoter operably linked to a first DNA sequence and a 3' untranslated region, wherein the first DNA sequence encodes a germination inhibitor; and

(ii) an inducible promoter operably linked to a second DNA sequence and a 3' untranslated region, wherein the second DNA sequence encodes a germination restorer.

22. The plant, plant cell or seed of claim 21, wherein the germination inhibitor encodes a protein for inhibiting germination.

23. The plant, plant cell or seed of claim 21, wherein the germination inhibitor is an antisense molecule, a co-suppression molecule containing sequences homologous to endogenous gene sequences, or a ribozyme, in each case capable of inhibiting the level or function of an endogenous mRNA or protein.

24. The plant, plant cell or seed of claim 23, wherein the endogenous mRNA encodes a protein necessary for plant lipid mobilization.

25. The plant, plant cell or seed of claim 24, wherein the endogenous mRNA encodes an acyl CoA oxidase enzyme.

26. The plant, plant cell or seed of claim 25, wherein the endogenous mRNA encodes a soybean acyl CoA oxidase I (SEQ ID NO.7) or acyl CoA oxidase II (SEQ ID NO.8) mRNA.

27. The plant, plant cell or seed of claim 21, wherein the promoter is a CaMV 35S, FMV 35S, ubiquitin, actin, cyclophilin, isocitrate lyase, malate synthase, SIP-seedling inhibition protein, cysteine endopeptidase, tobacco class I ß-1,3-glucanase B, canola CA25, canola CA8, canola AX92, lipid transfer protein or rice serine carboxypeptidase promoter.

28. The plant, plant cell or seed of claim 21, wherein the germination restorer comprises a protein that allows the seed to germinate.

29. The plant, plant cell or seed of claim 28, wherein said compound is a yeast POX4 or yeast POX5 enzyme.

30. The plant, plant cell or seed of claim 21, wherein the germination inhibitor comprises an antisense RNA molecule capable of inhibiting the expression of the first DNA molecule.

31. The plant, plant cell or seed of claim 21, wherein the inducible promoter is derived from the 27Kd subunit of glutathione-S-transferase II, GH2/4, amylase, HVA22,

tetracycline A promoter/operator, *E. coli* lac promoter/operator, glucocorticoid, inducible promoter element, copper inducible promoter element or nitrate inducible promoter element.

32. The plant, plant cell or seed of claim 23, wherein the germination restorer comprises a protein that is functionally equivalent *in planta* to the protein necessary for germination which is inhibited by the germination inhibitor.

33. A recombinant DNA molecule comprising a promoter operably linked to a DNA sequence which encodes an RNA molecule which is complementary to the endogenous RNA selected from the group consisting of soybean acyl CoA oxidase I (SEQ ID NO.7) and acyl CoA oxidase II (SEQ ID NO.8) mRNA, operably linked to a 3' untranslated region.

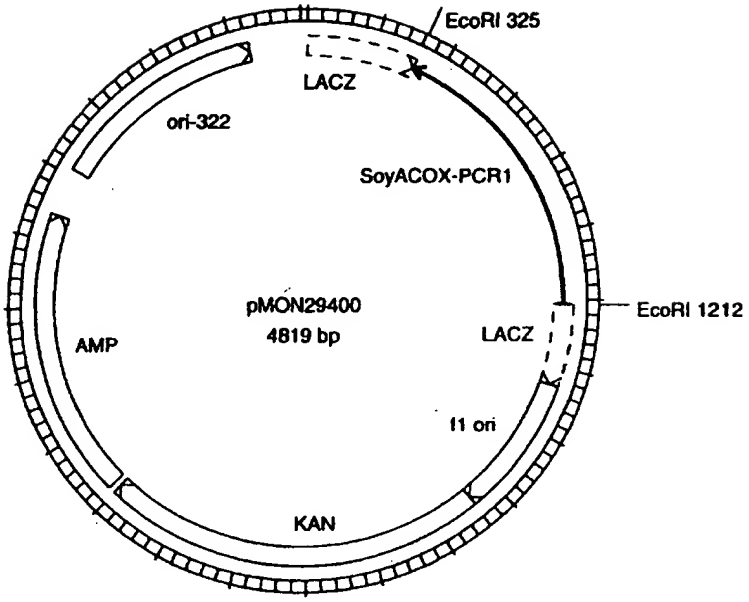
34. The DNA molecule of claim 33, wherein the promoter is a CaMV 35S, FMV 35S, ubiquitin, actin, cyclophilin, isocitrate lyase, malate synthase, SIP-seedling inhibition protein, cysteine endopeptidase, tobacco class I ß-1,3-glucanase B, canola CA25, canola CA8, canola AX92, lipid transfer protein or rice serine carboxypeptidase promoter.

35. A recombinant DNA molecule comprising a promoter operably linked to a DNA sequence which causes the production of a yeast acyl CoA Oxidase enzyme, operably linked to a 3' untranslated region.

36. The DNA molecule of claim 35, wherein said enzyme is the yeast POX4 or yeast POX5 enzyme.

37. The DNA molecule of claim 35, wherein the promoter is derived from the 27Kd subunit of glutathione-S-transferase II, GH2/4, amylase, HVA22, tetracycline A promoter/operator, *E. coli* lac promoter/operator, glucocorticoid inducible promoter element, copper inducible promoter element or nitrate inducible promoter element.

38. An isolated double stranded DNA molecule comprising a contiguous sequence of SEQ ID NO.7 or SEQ ID NO.8.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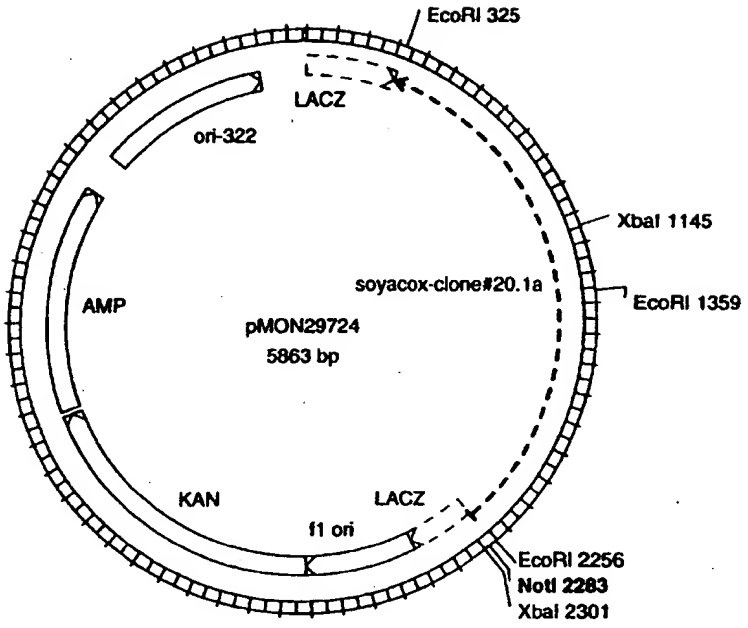
LACZ
ori-322
SoyACOX-PCR1
pMON29400
4819 bp
AMP
LACZ
EcoRI 1212
f1 ori
KAN

Figure 1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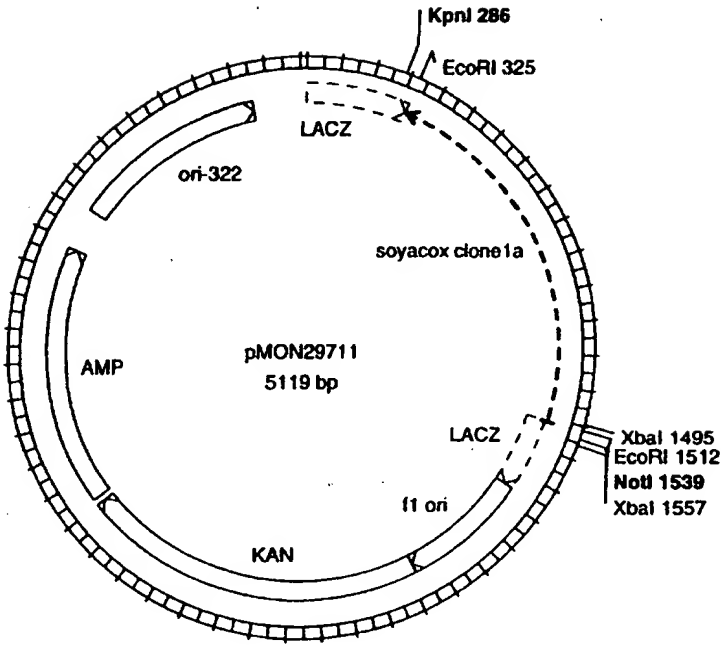
LACZ
ori-322
XbaI 1145
soyacox-clone#20.1a
EcoRI 1359
AMP
pMON29724
5863 bp
KAN
LACZ
f1 ori
EcoRI 2256
NotI 2283
XbaI 2301

Figure 2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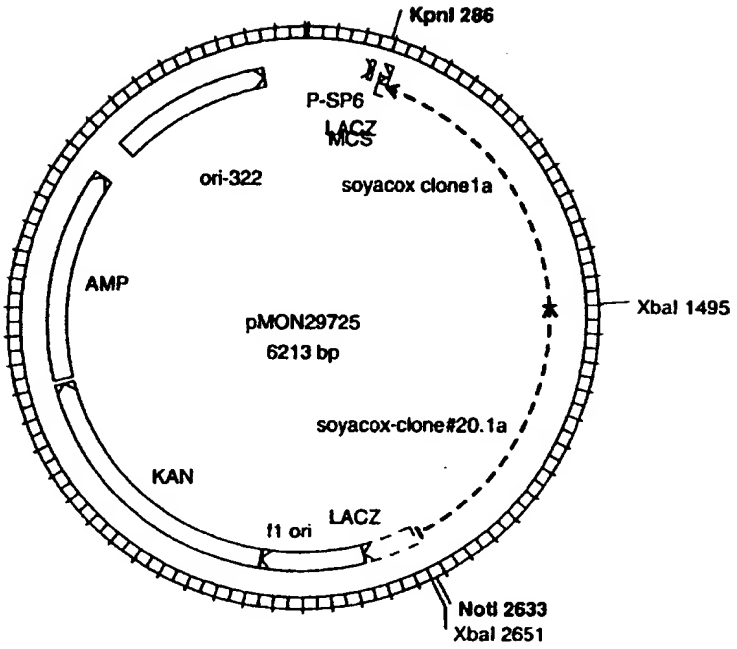
LACZ
ori-322
soyacox clone1a
AMP
pMON29711
5119 bp
LACZ
XbaI 1495
EcoRI 1512
NotI 1539
XbaI 1557
f1 ori
KAN

Figure 3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P-SP6
LACZ
MCS
ori-322
soyacox clone1a
AMP
XbaI 1495
pMON29725
6213 bp
soyacox-clone#20.1a
KAN
f1 ori
LACZ
NotI 2633
XbaI 2651

Figure 4

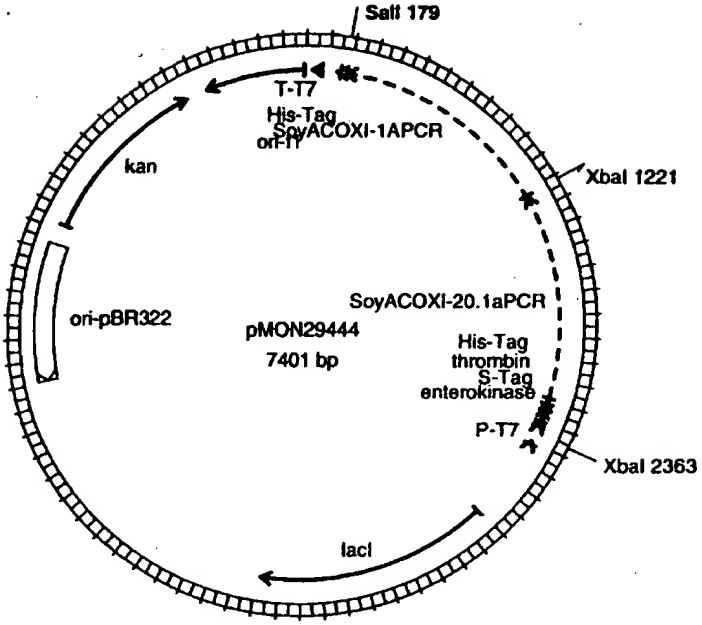

SalI 179
T-T7
His-Tag
SoyACOXI-1APCR
ori-f1
kan
XbaI 1221
ori-pBR322
SoyACOXI-20.1aPCR
pMON29444
7401 bp
His-Tag
thrombin
S-Tag
enterokinase
P-T7
XbaI 2363
lacI

Figure 5

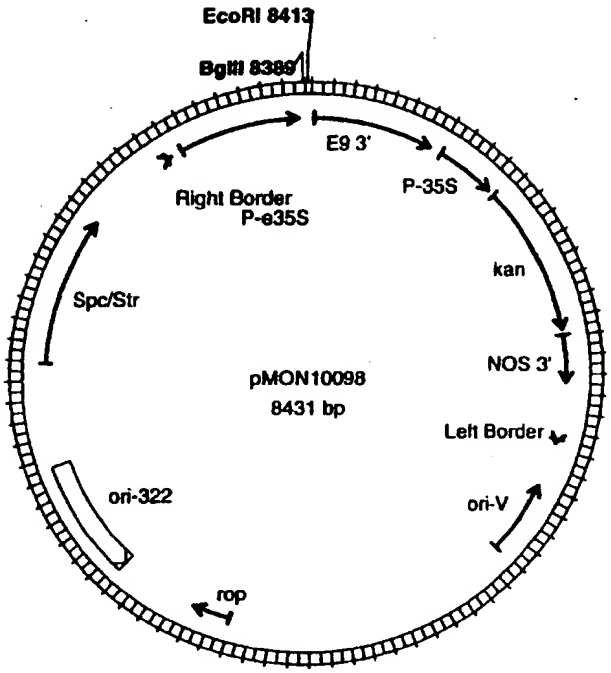
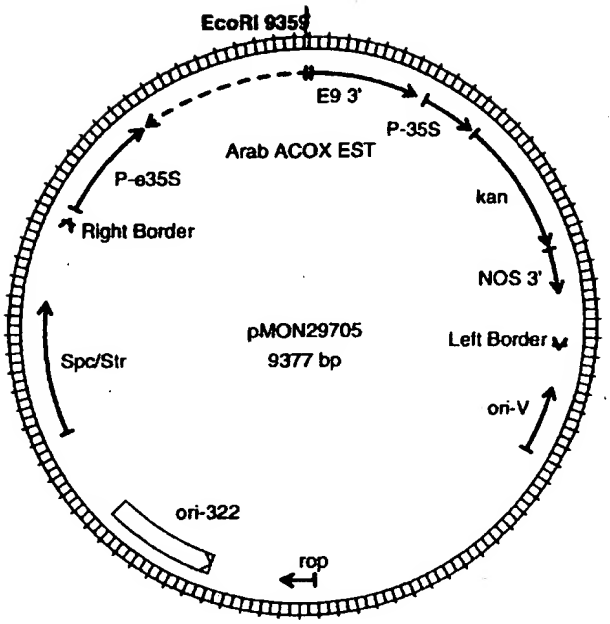

EcoRI 8413
BglII 8389
E9 3'
P-35S
Right Border
P-e35S
kan
Spc/Str
NOS 3'
pMON10098
8431 bp
Left Border
ori-322
ori-V
rop

Figure 6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E9 3'
P-35S
Arab ACOX EST
P-e35S
kan
Right Border
NOS 3'
pMON29705
9377 bp
Left Border
Spc/Str
ori-V
ori-322
rop

Figure 7

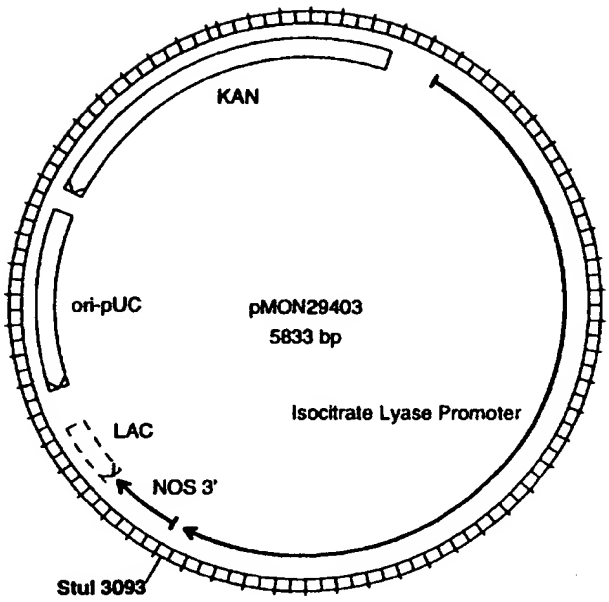

KAN
ori-pUC
pMON29403
5833 bp
Isocitrate Lyase Promoter
LAC
NOS 3'
StuI 3093

Figure 8

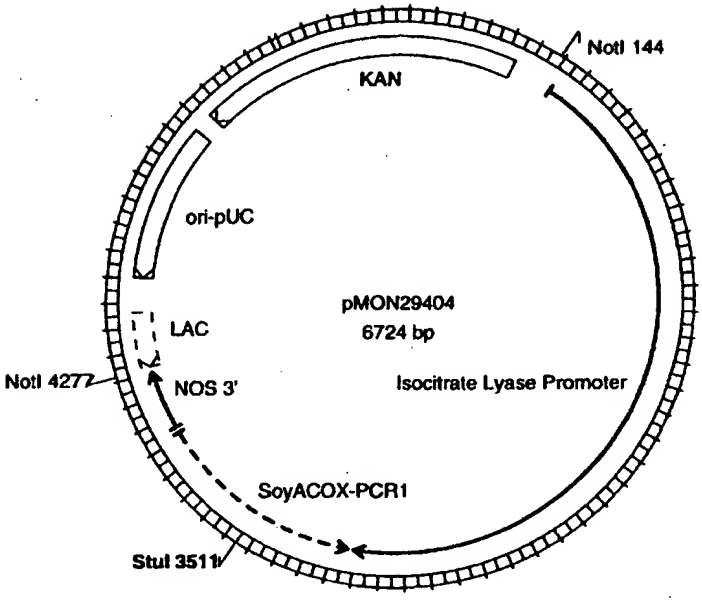

NotI 144
KAN
ori-pUC
pMON29404
6724 bp
LAC
NotI 4277
NOS 3'
Isocitrate Lyase Promoter
SoyACOX-PCR1
StuI 3511

Figure 9

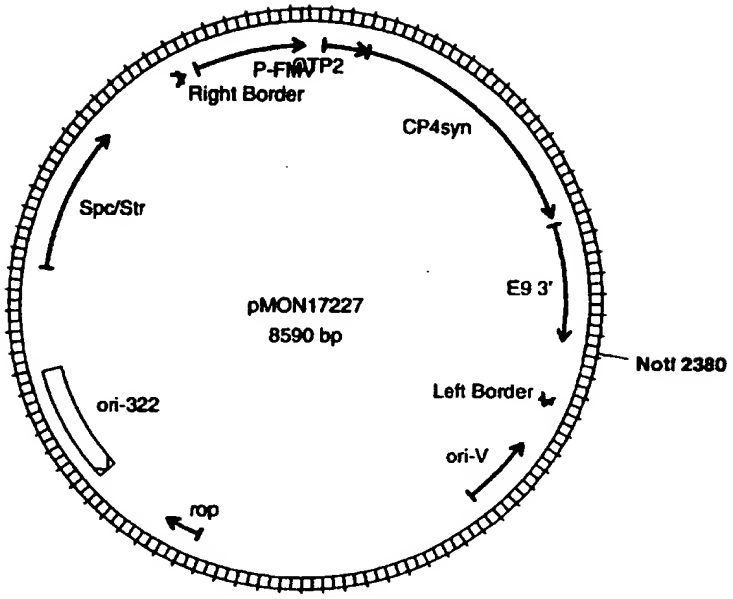
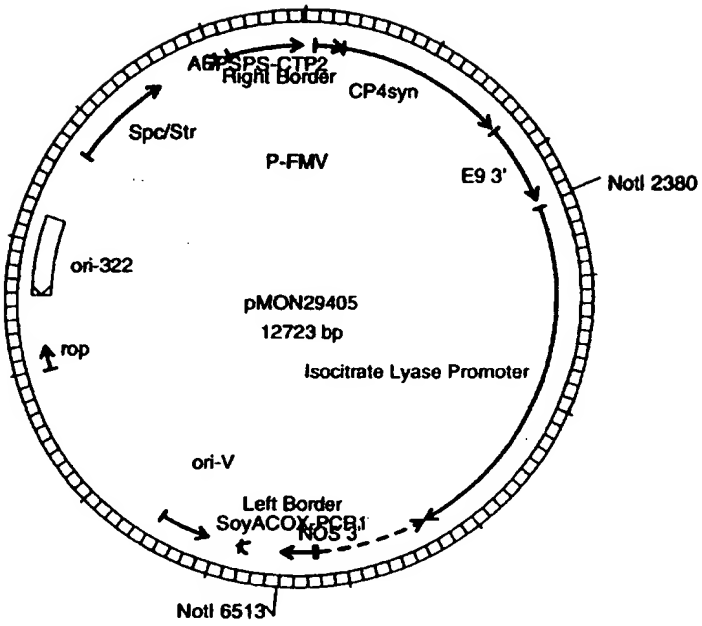

P-FMV
CTP2
Right Border
CP4syn
Spc/Str
E9 3'
pMON17227
8590 bp
NotI 2380
Left Border
ori-322
ori-V
rop

Figure 10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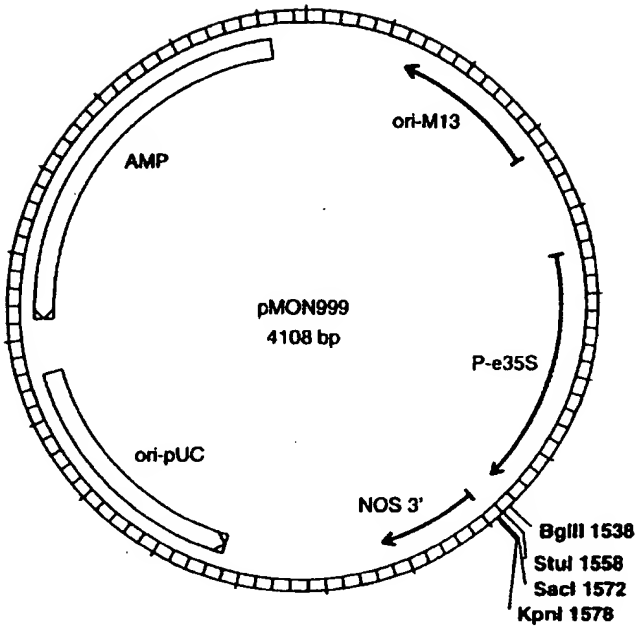
Right Border
CP4syn
Spc/Str
P-FMV
E9 3'
NotI 2380
ori-322
pMON29405
12723 bp
rop
Isocitrate Lyase Promoter
ori-V
Left Border
SoyACOX-PCB1
NOS 3'
NotI 6513

Figure 11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P-e35S
ori-pUC
NOS 3'
BglII 1538
StuI 1558
SacI 1572
KpnI 1578

Figure 12

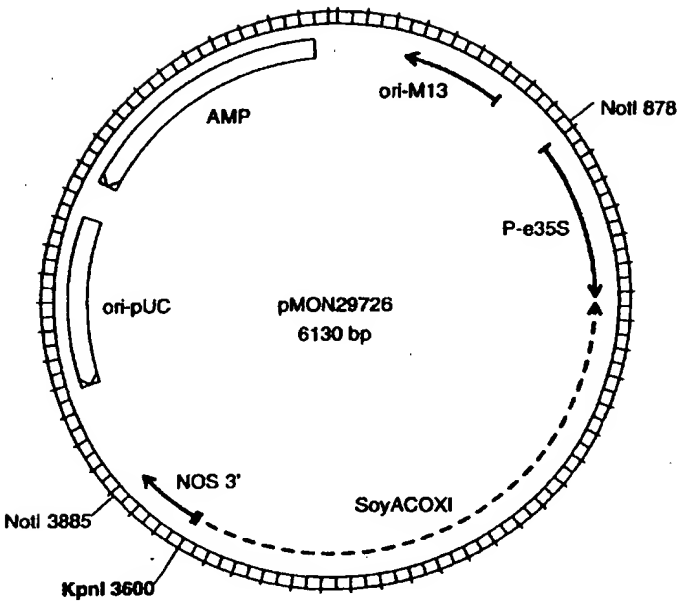
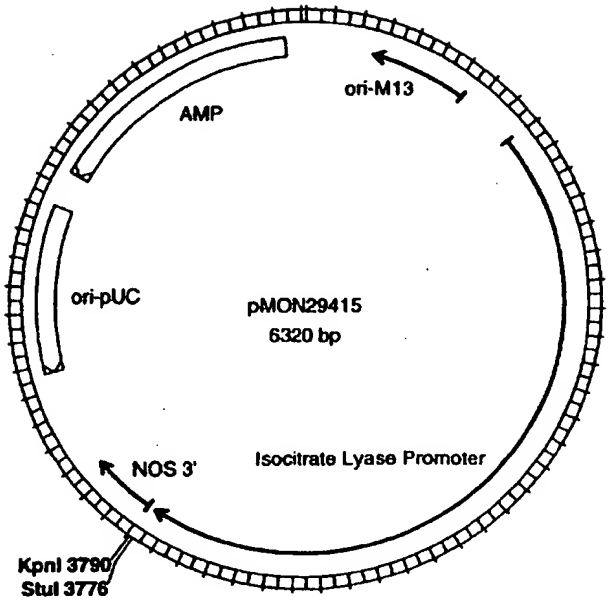

ori-M13
NotI 878
AMP
P-e35S
ori-pUC
pMON29726
6130 bp
NOS 3'
SoyACOXI
NotI 3885
KpnI 3600

Figure 13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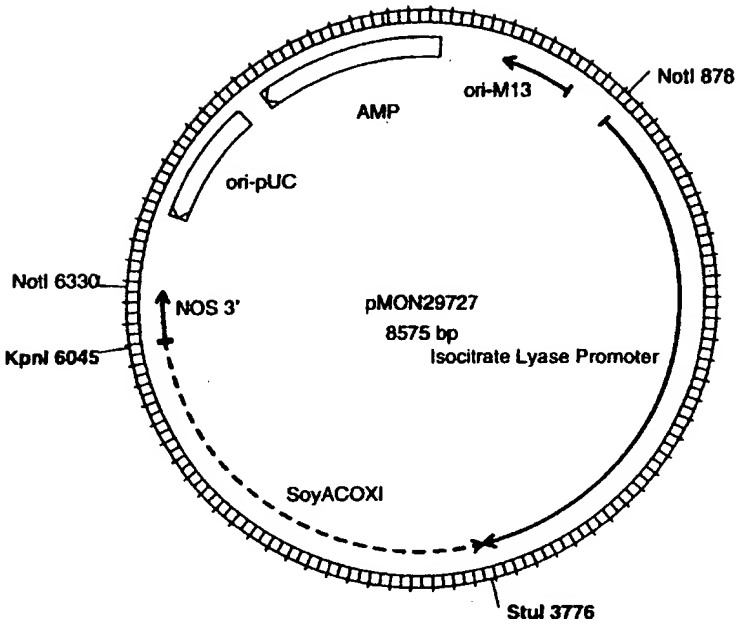
NOS 3'
Isocitrate Lyase Promoter
KpnI 3790
StuI 3776

Figure 14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ori-M13
AMP
ori-pUC
NotI 6330
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pMON29727
8575 bp
Isocitrate Lyase Promoter
KpnI 6045
SoyACOXI
StuI 3776

Figure 15

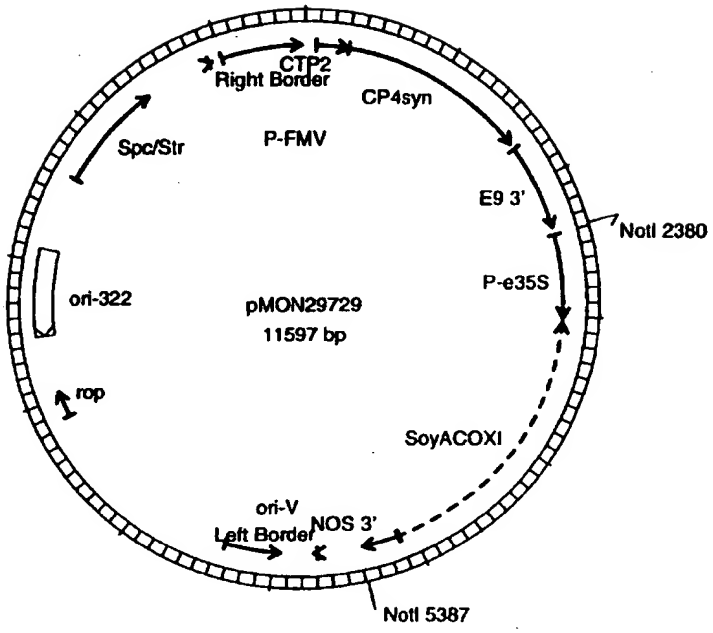
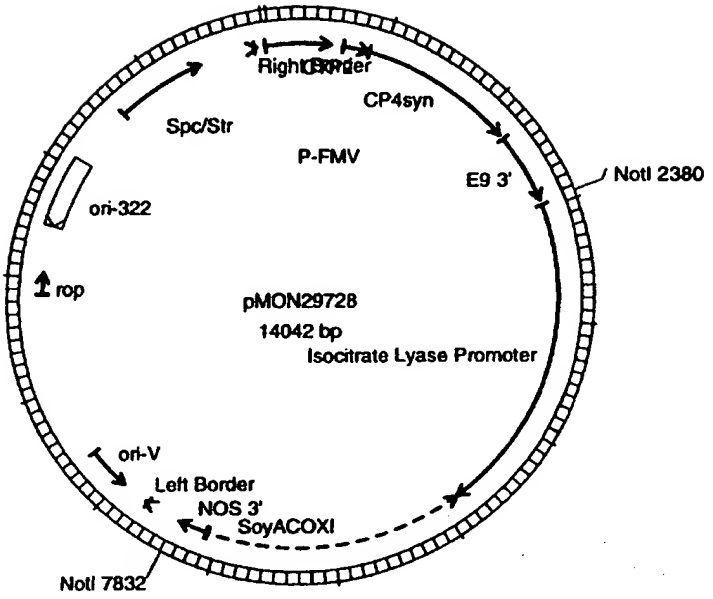

CTP2
Right Border
CP4syn
Spc/Str
P-FMV
E9 3'
NotI 2380
P-e35S
ori-322
pMON29729
11597 bp
rop
SoyACOXI
ori-V
Left Border
NOS 3'
NotI 5387

Figure 16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Spc/Str
P-FMV
E9 3'
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rop
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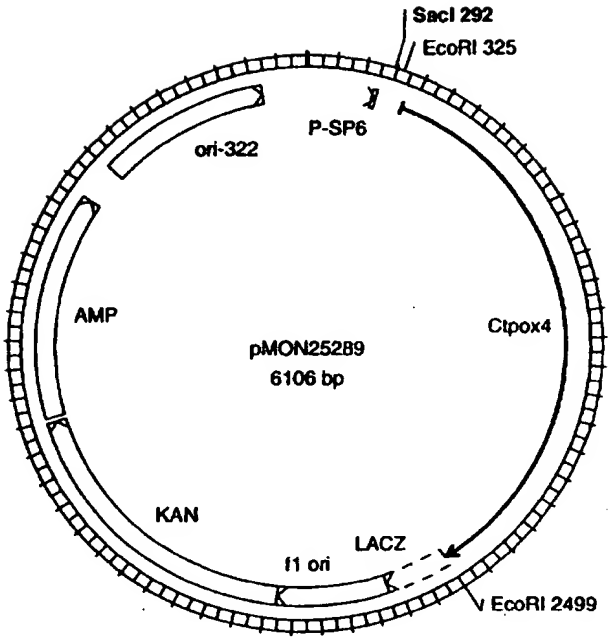
14042 bp
Isocitrate Lyase Promoter
ori-V
Left Border
NOS 3'
SoyACOXI
NotI 7832

Figure 17


SacI 292
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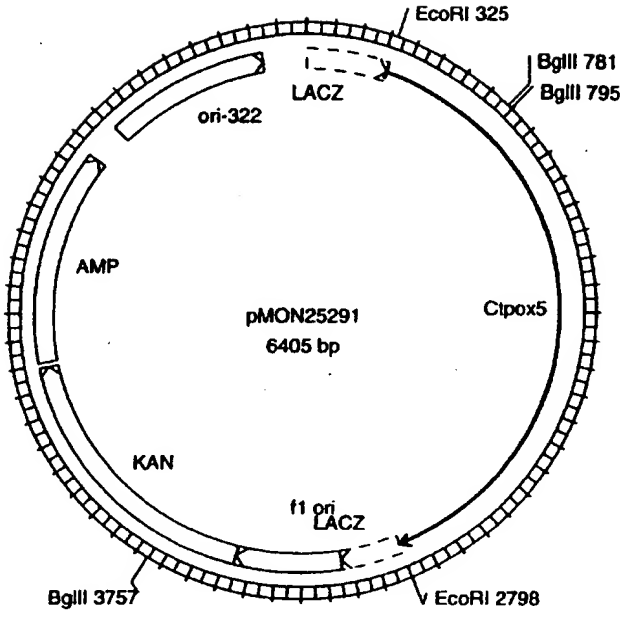
6106 bp
KAN
LACZ
f1 ori
EcoRI 2499

Figure 18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BglII 781
BglII 795
LACZ
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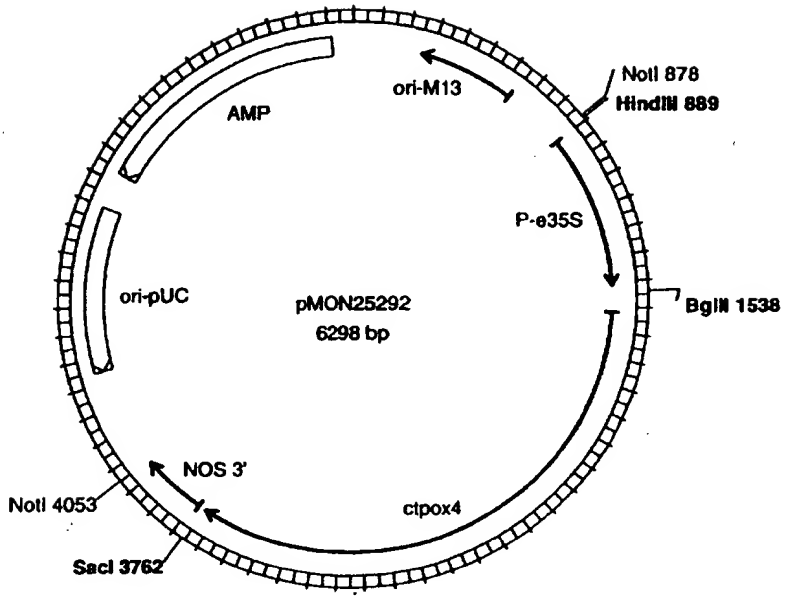
Ctpox5
KAN
f1 ori
LACZ
BglII 3757
V EcoRI 2798

Figure 19


NotI 878
HindIII 889
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AMP
P-e35S
ori-pUC
pMON25292
6298 bp
BglII 1538
NOS 3'
NotI 4053
ctpox4
SacI 3762

Figure 20

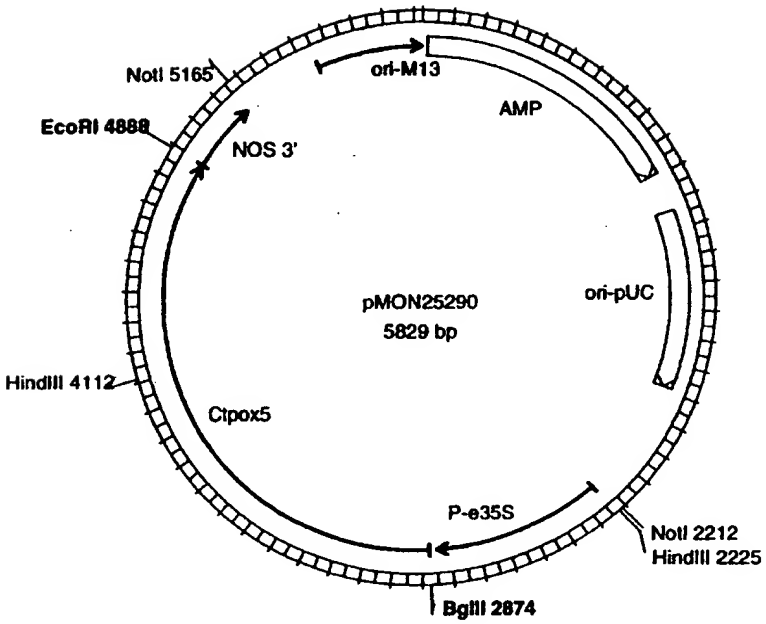
NotI 5165
ori-M13
AMP
EcoRI 4888
NOS 3'
pMON25290
5829 bp
ori-pUC
HindIII 4112
Ctpox5
P-e35S
NotI 2212
HindIII 2225
BglII 2874

Figure 21

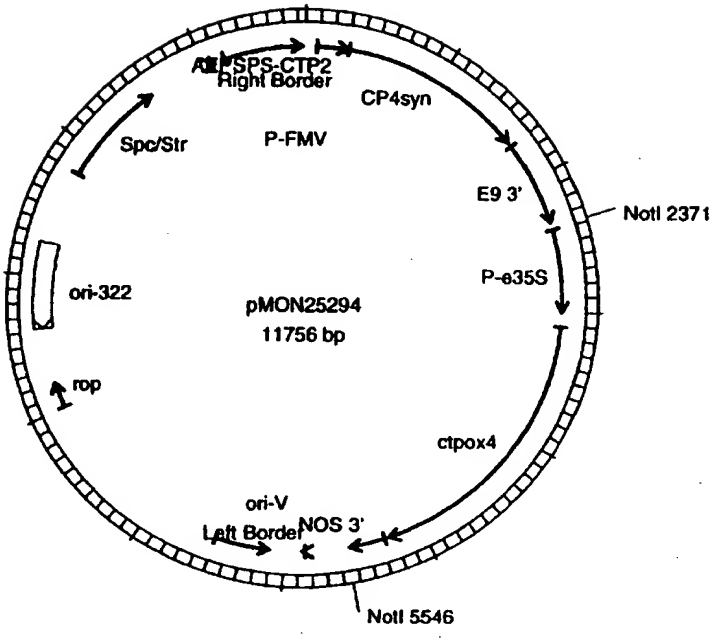
AS1/SPS-CTP2
Right Border
CP4syn
Spc/Str
P-FMV
E9 3'
NotI 2371
P-e35S
ori-322
pMON25294
11756 bp
rop
ctpox4
ori-V
Left Border
NOS 3'
NotI 5546

Figure 22

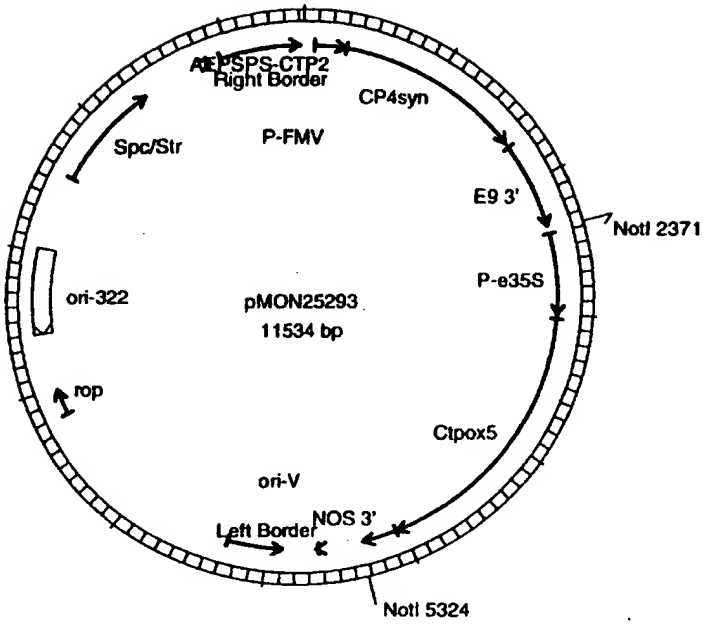
AtEPSPS-CTP2
Right Border
CP4syn
Spc/Str
P-FMV
E9 3'
NotI 2371
P-e35S
ori-322
pMON25293
11534 bp
rop
Ctpox5
ori-V
NOS 3'
Left Border
NotI 5324

Figure 23

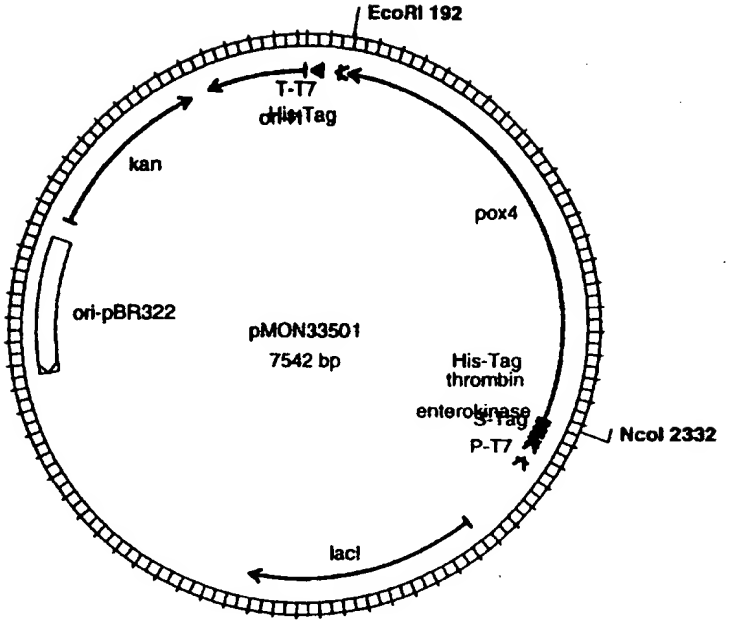
EcoRI 192
T-T7
HisTag
kan
pox4
ori-pBR322
pMON33501
7542 bp
His-Tag
thrombin
enterokinase
S-Tag
P-T7
NcoI 2332
lacI

Figure 24

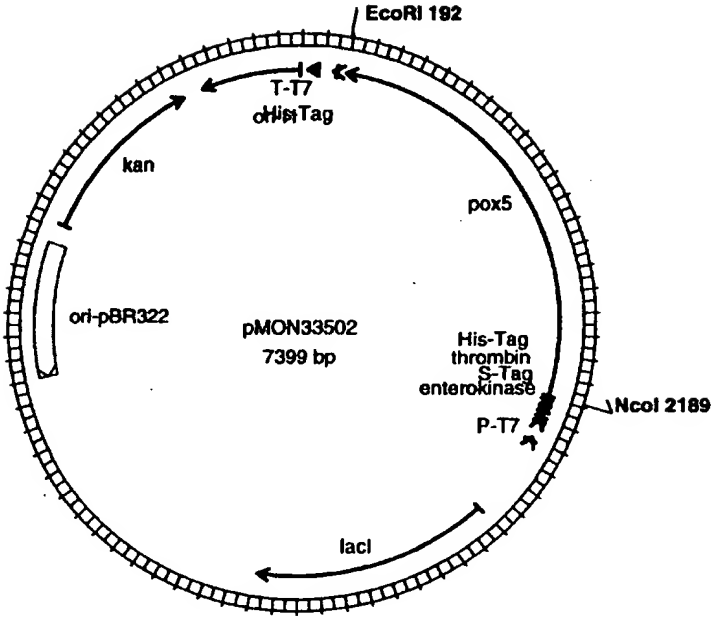
EcoRI 192
T-T7
His-Tag
kan
pox5
ori-pBR322
pMON33502
7399 bp
His-Tag
thrombin
S-Tag
enterokinase
NcoI 2189
P-T7
lacI

Figure 25

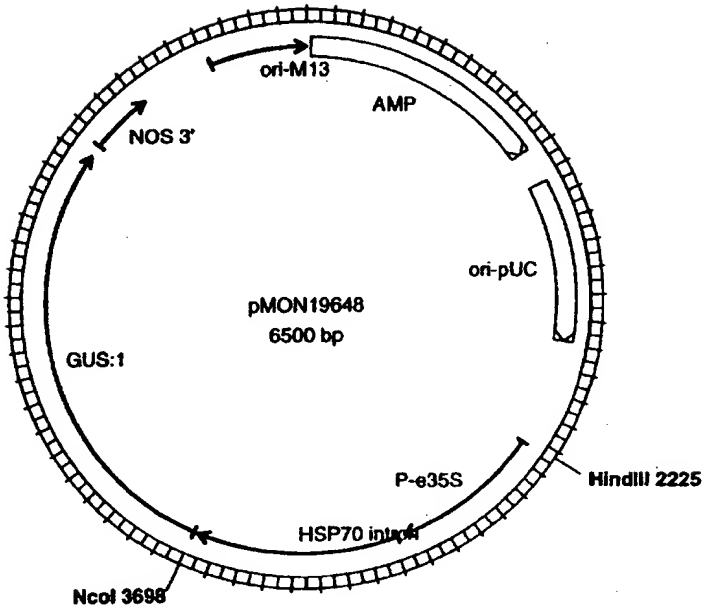
ori-M13
AMP
NOS 3'
ori-pUC
pMON19648
6500 bp
GUS:1
P-e35S
HindIII 2225
HSP70 intron
NcoI 3698

Figure 26

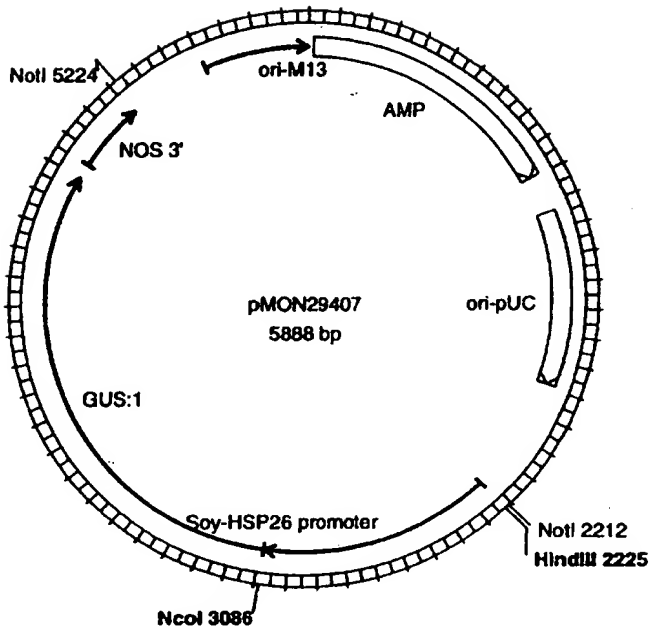
NotI 5224
ori-M13
AMP
NOS 3'
pMON29407
5888 bp
ori-pUC
GUS:1
Soy-HSP26 promoter
NotI 2212
HindIII 2225
NcoI 3086

Figure 27

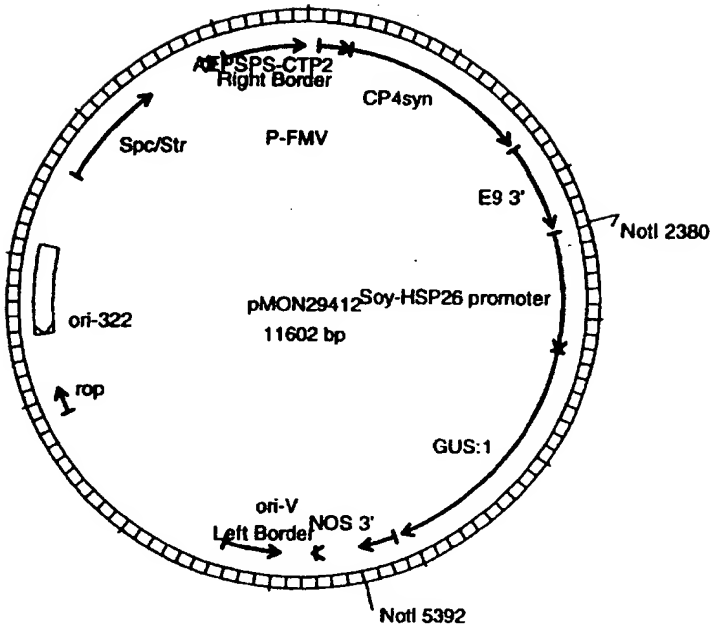
Right Border
CP4syn
Spc/Str
P-FMV
E9 3'
NotI 2380
pMON29412
11602 bp
Soy-HSP26 promoter
ori-322
rop
GUS:1
ori-V
Left Border
NOS 3'
NotI 5392

Figure 28

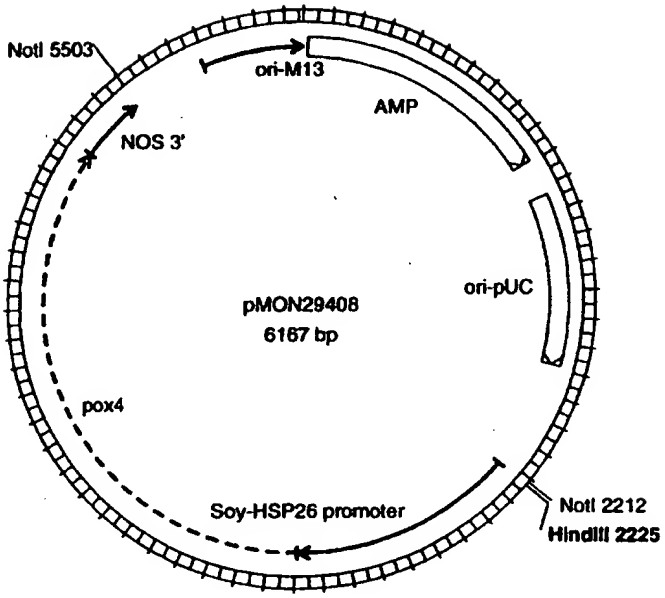
NotI 5503
ori-M13
AMP
NOS 3'
ori-pUC
pMON29408
6167 bp
pox4
Soy-HSP26 promoter
NotI 2212
HindIII 2225

Figure 29

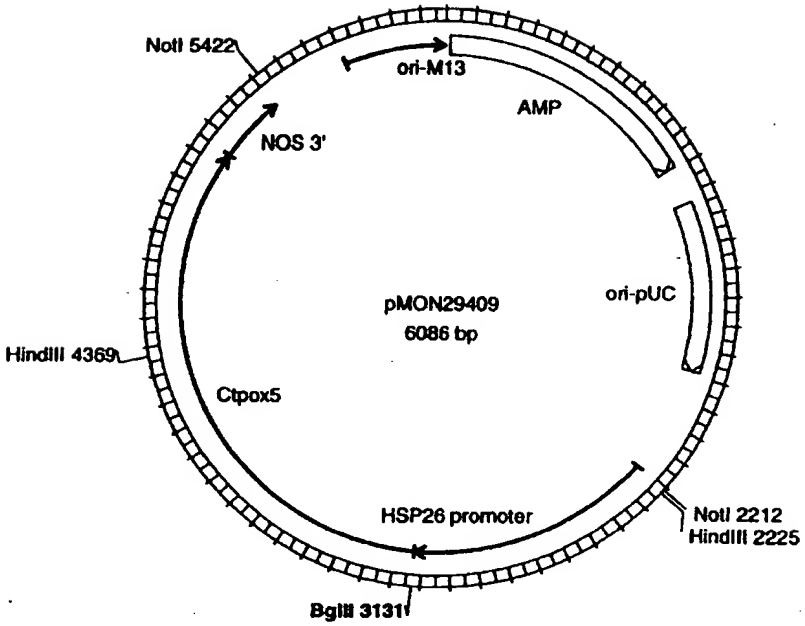
NotI 5422
ori-M13
AMP
NOS 3'
ori-pUC
pMON29409
6086 bp
HindIII 4369
Ctpox5
HSP26 promoter
NotI 2212
HindIII 2225
BglII 3131

Figure 30

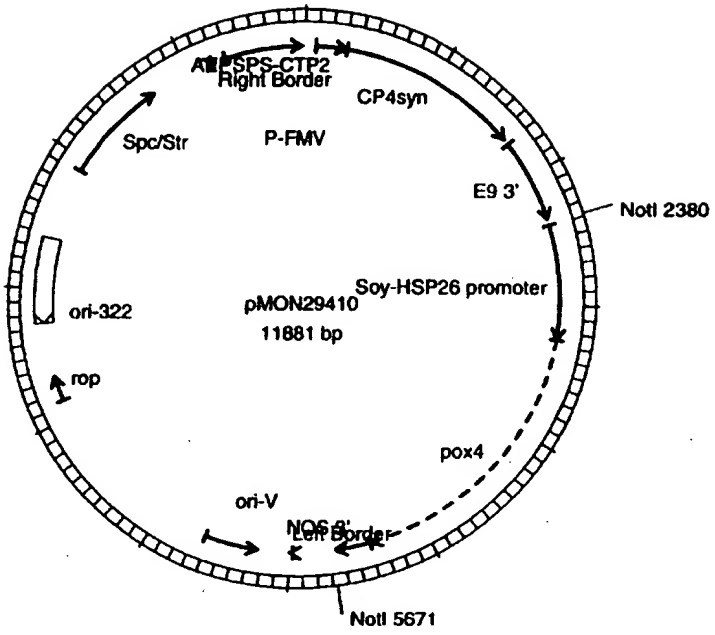
AtEPSPS-CTP2
Right Border
CP4syn
Spc/Str
P-FMV
E9 3'
NotI 2380
Soy-HSP26 promoter
ori-322
pMON29410
11881 bp
rop
pox4
ori-V
NOS 5'
Left Border
NotI 5671

Figure 31

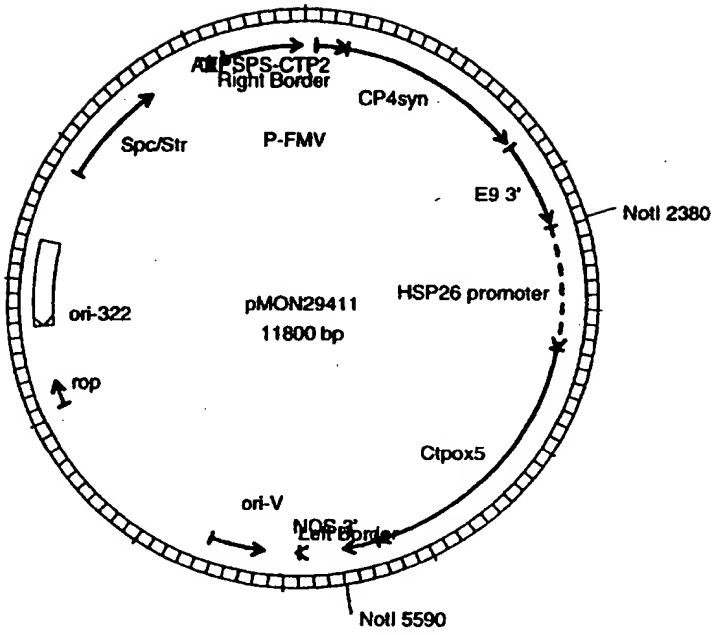
AtEPSPS-CTP2
Right Border
CP4syn
Spc/Str
P-FMV
E9 3'
NotI 2380
ori-322
pMON29411
11800 bp
HSP26 promoter
rop
Ctpox5
ori-V
NOS 3'
Left Border
NotI 5590

Figure 32

INTERNATIONAL SEARCH REPORT

Interr nal Application No
PCT/US 97/08732

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/53 C12N15/82 C12N15/11 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SMALL G. ET AL.: "Acyl-CoA oxidase contains two targeting sequences each of which can mediate protein import into peroxisomes" THE EMBO JOURNAL, vol. 7, no. 4, April 1988, pages 1167-1173, XP002041280 see the whole document ---	35-37
X	WO 94 03619 A (ZENECA LTD ;BRIGHT SIMON WILLIAM JONATHAN (GB); GREENLAND ANDREW J) 17 February 1994 cited in the application see the whole document --- -/--	1-3,7,8, 10,11, 13-15, 19, 21-23, 27,28, 30,31

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
22 September 1997	01. 10. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	Kania, T

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INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCI/US 97/08732

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 93 15213 A (ZENECA LTD) 5 August 1993 see the whole document ---	1-38
A	WO 96 04393 A (DELTA AND PINE LAND COMPANY ;US GOVERNMENT (US)) 15 February 1996 see the whole document -----	1-38

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INTERNATIONAL SEARCH REPORT

Interr al Application No
PCT/US 97/08732

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		EP 0658207 A	21-06-95
WO 9315213 A	05-08-93	AU 3362293 A	01-09-93
WO 9604393 A	15-02-96	AU 3205095 A	04-03-96
		EP 0775212 A	28-05-97
		ZA 9506410 A	11-03-96